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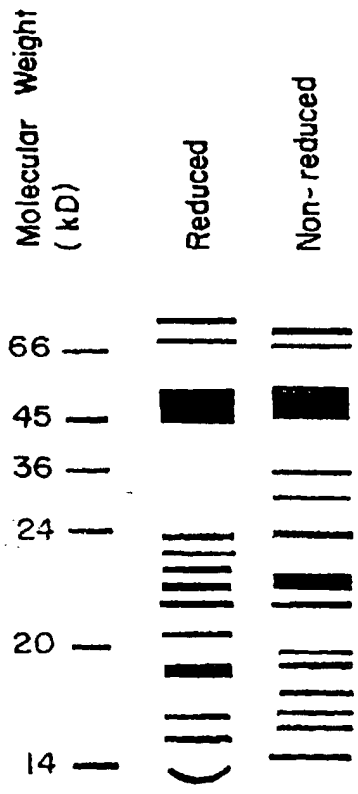
(54) Title: PROTEIN MIXTURES FOR WOUND HEALING



(57) Abstract: A protein mixture that is useful in the treatment of wounds, where the mixture is isolated from bone or is produced from recombinant proteins and may include two or more of BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, TGF-β1, TGF-β2, TGF-β3, and FGF-1.

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Protein Mixtures for Wound Healing

Description

Background Art

The invention relates to use of protein mixtures, comprising a variety of growth factors, for use in the treatment of wounds.

Wound healing is a complex process involving several cell types and growth factors for an effective closure. The normal wound healing process can be broadly classified into three stages namely the inflammatory, proliferative and maturation phases. The inflammatory phase lasts 0-2 days and involves an orderly recruitment of cells to the wound area. This is followed by the 2-6 day proliferative phase, in which fibroblasts, keratinocytes and other cells in the wound bed begin to actively proliferate to close the wound. The maturation phase follows the proliferative phase, peaking at 21 days, by which time the wound is completely healed by restructuring the initial scar tissue.

A problematic wound does not follow the normal timetable for the healing process as described above. A problematic wound could fail to follow the normal healing process for any number of reasons, including nutrition, vascular status, metabolic factors, age, immune status, drug therapy, neurologic status and psychologic status, among others. Several local factors also play an important role in wound healing, including the presence of necrotic tissue in the area, infection, foreign body presence, degree of desiccation, presence of edema, pressure, friction, shear maceration and dermatitis.

It has been shown from wound fluid composition studies that growth factors play an important role in all three phases of wound healing. The cell types that are recruited to the wound area secrete growth factors that assist in and promote the wound healing process. Platelets, for example, are the first cell type to be recruited at the wound site, and initiate the wound healing process by secreting growth factors (i.e., platelet derived growth factors, or PDGF) which are chemotactic for other cell types. By so doing, the platelets assist in the recruitment and proliferation of additional cell types that promote synthesis of new tissue. In addition to the above mentioned functional properties, growth factors also have the ability to regulate protein synthesis within the cell and control intracellular signaling thus allowing cells to communicate with one another.

Since wound healing is a complex process, which involves formation of connective tissue, and new blood vessels to nourish the site, it is evident that several growth factors

come into play. In chronic wounds there is an increase in collagenase activity and higher levels of inflammatory cytokines. Additionally, there is an absence of growth factors in the wound fluid, which causes the cells to be mitotically incompetent. All of these factors cause impaired wound healing. Some of these factors have been studied in the preclinical animal models as well as in the clinic. Most growth factor studies involving the wound healing process involve tests in the 20-25 day range, which appears to adequately model the normal wound healing process. However, it is now realized that to get 100% closure of problematic wounds, longer study periods such as long as 6 months or more would be advantageous.

The only FDA approved growth factor for wound healing use in the clinic is platelet derived growth factor (PDGF) marketed by Ortho-McNeil Pharmaceutical as REGRANEX(r). REGRANEX(r) contains becaplermin, a recombinant human platelet-derived growth factor (rhPDGF-BB) for topical administration. Becaplermin is produced by recombinant DNA technology by insertion of the gene for the B chain of platelet derived growth factor (PDGF) into yeast. Becaplermin has a molecular weight of approximately 25 KD and is a homodimer composed of two identical polypeptide chains that are bound together by disulfide bonds. REGRANEX(r) is a non-sterile, low bioburden, preserved, sodium carboxymethylcellulose-based (CMC) topical gel, containing the active ingredient becaplermin and the inactive ingredients sodium chloride, sodium acetate trihydrate, glacial acetic acid, water for injection, and methylparaben, propylparaben, and m-cresol as preservatives and l-lysine hydrochloride as a stabilizer.

Studies of various growth factors in the wound healing process have been conducted. Some of the findings from these studies are summarized below:

1) PDGF-BB (the growth factor in REGRANEX(r)) is a chemoattractant for neutrophils, monocytes, and fibroblasts. In wound healing applications it has been shown to increase extracellular matrix deposition and enhance proliferation of fibroblasts. PDFG is not an angiogen, however. Thus, additional growth factors will be required for the healthy maintenance of neodermis.

2) Fibroblast Growth Factor (FGF) increases capillary density and proliferation of fibroblasts. A topical application in gel form was tested and it was shown that there was no systemic absorption of the protein (< 1% of the dose detected).

3) Transforming growth factor ß-2 (TGF ß-2) is a growth factor that enhances proliferation of several cell types both in vitro and in vivo and has been tested in venous ulcer healing and in diabetic foot ulcer trials. In a two-arm clinical study a 40% reduction

of wound size compared to the control wound was observed in 6 weeks when used at 0.5 μg/cm2. However, in a 3 arm clinical study when 2.5 μg/cm2 was tested for comparison against standard XEROFORM(tm) dressing, the results were not very encouraging.

4) Epidermal growth Factor (EGF) produced by platelets and macrophages is a mitogen for epithelial cells. This growth factor was first tested in burn patients and the initial results were promising. However, when tested in volunteers there was no difference between growth factor treatments and placebo. This could be due to the fact that EGF is not good for migration of keratinocytes, but is a good mitotic agent.

5) Keratinocyte Growth Factor-2 (KGF-2) was tested for its ability to increase ephithelialization. By day 6 the interstices were closed. KGF-2 promotes re-epithelialization in young and old animals suggesting indirect mechanisms for neo-granulation tissue formation. Xia Y.D., et al., J. Pathol. (1999) 188: 431-438. There is increased resistance to mechanical stress of healed wounds; hence KGF-2 may be useful for the treatment of surgical wounds. Jiminez, P.A. & Rampy, M.A., (1999) J. Surg. Res. 81: 238-242.

6) Connective tissue growth factor (CTGF) is a secreted, mitogenic, chemotactic and cell matrix inducing factor encoded by an immediate early growth responsive gene. Involvement of CTGF in human atherosclerosis and fibrotic disorders suggests a role in tissue regeneration like wound repair, but also in aberrant deposition of extracellular matrix. In fact, anti-CTFG antibodies have been used to block the fibrotic cascade.

Studies on the kinetics of action of various growth factors demonstrated that some growth factors such as granulocyte-monocyte colony stimulating factor (GMCSF) and bovine FGF acted sequentially. It was hypothesized that a combination of growth factors would be better than a single growth factor treatment. However, in animal models, a combination of these two factors actually slowed the regenerative process and healing never achieved 100%. Hence, sequential delivery of these factors was attempted: GMCSF was administered first followed by FGF delivery 25 days later. In a single study, no improvement over control could be demonstrated.

In yet another study combining TGF-ß, bFGF (basic FGF) and CTGF it was found that TGF-ß1, TGF-ß2 or TGF-ß3 caused skin fibrosis after 3 days of continuous injection but the change was transient and disappeared after 7 days of continuous injection. In contrast, irreversible fibrosis was observed upon simultaneous injection of TGF-ß and bFGF or TGF-ß and CTGF, or TGF-ß injection for the first 3 days followed by bFGF or

CTGF injection for the next 4 days. These observations suggest that TGF-ß1 induces skin fibrosis and bFGF or CTGF maintains it in various skin fibrotic disorders.

Another way of obtaining growth factor mixtures considered the use of platelet releasate, which contains a collection of growth factors released from platelets derived from blood. The advantages of this material are that it is autologous or homologous, and is readily available and presumably contains the required factors in the proper ratio. To date, although some improvement in the healing process was observed initially, by 24 weeks there was no difference between growth factor and placebo treatments.

It is thus apparent that although several polypeptide growth factors have shown significant biological activity in pre-clinical wound repair models, the only growth factor that has proven to be effective in the clinic is the human recombinant PDGF-BB. This may be due to poor delivery, drug instability or the inability of a single factor to orchestrate the complex process of wound healing. An effective treatment should address issues such as angiogenesis, efficient collagen deposition and proper epithelialization to close the wound.

Summary of Invention

The invention comprises compositions and methods for improving the wound healing process in living animals, including human subjects. In preferred embodiments, the invention comprises a mixture of growth factors, which improve the wound healing process. In this context, the terms "excluding," "exclusion," or "excluded" refers to the removal of substantially all of an indicated component, to the extent that such component can be removed from a mixture with inmmunoaffinity chromatography or otherwise not included in the mixture. The term "pharmaceutically acceptable carrier" is used herein in the ordinary sense of the term and includes all known carriers including water.

"BP" is a protein cocktail derived from bone as described in U.S. Patent Nos. 5,290,763, 5,371,191, and 5,563,124 (each of which is hereby incorporated by reference herein in its entirety). In brief, the cocktail is prepared by guanidine hydrochloride protein extraction of demineralized bone particles. The extract solution is filtered, and subjected to a two step ultrafiltration process. In the first ultrafiltration step an ultrafiltration membrane having a nominal molecular weight cut off (MWCO) of 100 kD is employed. The retentate is discarded and the filtrate is subjected to a second ultrafiltration step using an ultrafiltration membrane having a nominal MWCO of about 10 kD. The retentate is then subjected to diafiltration to substitute urea for guanidine. The protein-containing urea solution is then subjected to sequential ion exchange chromatography, first anion exchange chromatography followed by cation exchange chromatography. The osteoinductive proteins

produced by the above process are then subjected to HPLC with a preparative VYDAC(tm) column at and eluted with shallow increasing gradient of acetonitrile. One minute fractions of the HPLC column eluate are pooled to make the BP cocktail (fraction number can vary slightly with solvent composition, resin size, volume of production lot, etc.). One embodiment of the BP cocktail is characterized as shown in Figures 1-6. Absolute and relative amounts of the growth factors present in the BP cocktail can be varied by collecting different fractions of the HPLC eluate. In a particularly preferred embodiment, fractions 29-34 are pooled. It is also contemplated that certain proteins may be excluded from the BP mixture without affecting wound healing activity.

BP was originally discovered as a mixture of proteins known to have osteogenic activity. However, it contains a plurality of growth factors and is strongly angiogenic. In particular, BP contains a number of bone morphogenetic proteins (BMPs), including BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, and BMP-7, as well as TGF-ß1, TGF-ß2, and TGF-ß3. FGF-1 is also present in the mixture. The presence of each of the foregoing proteins was detected using immunoblot techniques, as depicted Figure 14. When BP was tested in an animal model to determine if it would be effective in aiding wound closure, it was surprisingly discovered that BP promotes wound healing, even though it is a markedly different process than osteogenesis.

The protein compositions of the invention can be advantageously combined with traditional wound dressings including primary and secondary dressings, wet-to-dry dressings, absorbent dressings, nonadherent dressings, semipermeable dressings, transparent dressings, hydrocolloid dressings, hydrogels, foam dressings, alginate dressings, surgical tapes and the like as is appropriate for the type of wound being treated.

Compositions according to the present invention may also be combined with a variety of other active ingredients, such as aloe vera, arginine, glutamine, zinc, copper, vitamin C, B vitamins and other nutritional supplements, antibiotics, antiseptics, antifungals, deodorizers, and the like. Embodiments of the invention can also be combined with a variety of anti-inflammatory agents that inhibit the action of proinflammatory cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor-alpha. Many such inhibitors are well known, such as IL-1Ra, soluble TGF-ß receptor, cortocosteroids, and it is believed that more will be discovered in the future.

In one embodiment, the invention is a composition for the treatment of wounds comprising the proteins BMP-3 and TGF-ß2 in a pharmaceutically acceptable carrier. As shown in Figure 18, BMP-3 is the growth factor present in the highest concentration in the

BP mixture. TGF-ß2 is believed to play an important role in wound healing because it promotes the proliferation of several cell types, which is important, for example, in the proliferative stage of the wound healing process. As already noted, TGF-ß2 alone has been the subject of study as a wound healing agent. Without limitation as to specific mechanisms, it is believed that these two growth factors may be significant in the wound healing activity displayed by BP.

In another embodiment, compositions of the present invention comprise BMP-3, TGF-ß2, and one or more of BMP-2, BMP-4, BMP-5, BMP-6, and BMP-7 in a pharmaceutically acceptable carrier. BMP-6 is known to induce a cascade of events leading to the expression of both BMP-2 and BMP-4, both of which are known to have osteogenic activity. BMP-2 has also been implicated in the regulation of kidney tissue regeneration. BMP-7 (also known as OP-1) is currently undergoing preclinical testing as a wound-healing agent.

In still another embodiment, compositions of the present invention comprise BMP-3, TGF-ß2, one or more of BMP-2, BMP-4, BMP-5, BMP-6, and BMP-7, and one or more of FGF-1, TGF-ß1, and TGF-ß3. FGF-1 is known to be an angiogenic growth factor, although its activity is not as pronounced as FGF-2, which has not been detected in BP. TGF-ß1 and TGF-ß3 are both known to enhance cell proliferation.

The presence of a number of proteins, which are believed to have no growth factor activity has been detected in BP. Accordingly, these proteins, including histone proteins, ribosomal proteins, or both, may be excluded from compositions of the present invention. Alternatively, the composition may comprise the BP mixture isolated as described in U.S. Patent Nos. 5,290,763, 5,371,191, and 5,563,124 as shown in Figures 2 and 3 (lanes inside the box pooled to make BP). Histones and ribosomes may be excluded from the BP by, for example, antibody binding or other techniques known in the art. Additionally, the composition of matter may contain one or more of the listed active components supplied as a recombinantly produced protein. Preferably, the components are isolated from a natural source and are at least partially phosphorylated and glycosylated.

In another embodiment, the above compositions are used in wound healing applications together with a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier includes dressings such as hydrocolloid dressings, hydrogels, foam dressings, and alginate dressings. Additional active ingredients may include arginine, glutamine, zinc, copper, vitamin C, vitamin B1, vitamin B2, vitamin B3, vitamin B6, vitamin B12, and folate or growth factors such as epidermal growth factor, platelet derived

growth factor, insulin-like growth factor, keratinocyte growth factor, vascular endothelial growth factor, transforming growth factor alpha, nerve growth factor, connective tissue growth factor and granulocyte-monocyte colony stimulating factor. Inflammation inhibitor, such as interleukin-1 inhibitor, interleukin-6 inhibitor and tumor necrosis factor-alpha inhibitor may also be added to the composition. Of course, pain relief agents, disinfectants, antibiotics and other active ingredients suitable for particular wound applications may also be added thereto.

Brief Description of Drawings

Figure 1 illustrates an SDS-PAGE of a protein mixture according to the present invention, both in reduced and nonreduced forms.

Figure 2 is an SDS-PAGE gel of HPLC fractions 27-36 of a protein mixture according to an embodiment of the present invention.

Figure 3 is an SDS-PAGE gel with identified bands indicated according to the legend of Figure 4.

Figure 4 is an SDS-PAGE gel of a protein mixture according to an embodiment of the present invention with identified bands indicated, as provided in the legend.

Figure 5 is two-dimensional (2-D) SDS-PAGE gel of a protein mixture according to an embodiment of the present invention with internal standards indicated by arrows.

Figure 6 is a 2-D SDS-PAGE gel of a protein mixture according to an embodiment of the present invention with circled proteins identified as in the legend.

Figures 7A-O are mass spectrometer results for tryptic fragments from one-dimensional (1-D) gels of a protein mixture according to an embodiment of the present invention.

Figure 8 is a 2-D gel Western blot of a protein mixture according to an embodiment of the present invention labeled with anti-phosphotyrosine antibody.

Figures 9A-D are 2-D gel Western blots of a protein mixture according to an embodiment of the present invention, labeled with indicated antibodies. Figure 9A indicates the presence of BMP-3 and BMP-2. Figure 9B indicates the presence of BMP-3 and BMP-7. Figure 9C indicates the presence of BMP-7 and BMP-2, and Figure 9D indicates the presence of BMP-3 and TGF-ß1.

Figure 10 is a PAS (periodic acid schiff) stained SDS-PAGE gel of HPLC fractions of a protein mixture according to an embodiment of the present invention.

Figure 11is an anti-BMP-7 stained SDS-PAGE gel of a PNGase F treated protein mixture according to an embodiment of the present invention.

Figure 12 is an anti-BMP-2 stained SDS-PAGE gel of a PNGase F treated protein mixture according to an embodiment of the present invention.

Figures 13A-B are bar charts showing explant mass of glycosylated components in a protein mixture according to an embodiment of the present invention (Figure 13A) and ALP score (Figure 13B) of the same components.

Figure 14 is a chart showing antibody listing and reactivity.

Figures 15A-B together comprise a chart showing tryptic fragment sequencing data for components of a protein mixture according to an embodiment of the present invention.

Figures 16A-F together comprise a chart showing tryptic fragment mass spectrometry data for components of a protein mixture according to an embodiment of the present invention.

Figures 17A-B are an SDS-gel (Figure 17B) and a scanning densitometer scan (Figure 17A) of the same gel for a protein mixture according to an embodiment of the present invention.

Figure 18 is a chart illustrating the relative mass, from scanning densitometer quantification, of protein components in a protein mixture according to an embodiment of the present invention.

Figures 19A-D together comprise a chart showing mass spectrometry data of various protein fragments from 2D gels of a protein mixture according to an embodiment of the present invention.

Detailed Description of the Invention

EXAMPLE 1. BP IN SINGLE DOSE APPLICATION TO NUDE MICE

A single dose application of BP to full thickness wounds in nude mice covered with human meshed split thickness skin grafts has been found to heal the wound completely and faster than wounds not receiving the growth factor mixture. Although the specific manner in which the growth factors in BP affect the wound healing process is not fully understood, it is hypothesized that the synergistic action of the multiple growth factors present in BP helps the wounds recover better than those in control animals that have received the carrier alone.

Full thickness wounds were created in nude mice such that the wound area comprised about 20% of the total body surface. BP was prepared as in U.S. Patent Nos. 5,290,763, 5,371,191, and 5,563,124, and applied to the wound in a povidone carrier. The wound was then covered with human meshed split thickness skin grafts. The control group of animals received only the povidone carrier. The graft sites were dressed and closed with

band-aids to keep the dressing securely in place. The first dressing changes were carried out on day 5 post operative and every third day thereafter. The basic protocol is also described in "Clinical and Experimental Approaches to Dermal and Epidermal Repair: Normal and Chronic Wounds," pp. 429-442 (1991) Wiley-Liss, Inc. and Cooper M.L., et al., The Effects of Epidermal Growth factor and basic Fibroblast Growth factor on Epithelialization of Meshed Skin Graft Interstices, Prog. Clin. Biol. Res. (1991) 365: 429-42. Such protocols are known to persons of skill in the art.

The results were strongly encouraging. Single application of two concentrations (either 100 μg/wound site or 200 μg/wound site) of growth factor were tested. There was no difference either in the rate or degree of wound healing between the two groups. However, there was a marked difference between the group of animals that received the growth factor treatment and the control animals that did not receive the growth factor. By day 11 POD (post operative day), a 95% wound closure was observed in the animals that received the growth factor whereas the control animals showed only a 74% closure. By day 14 POD all growth factor treated animals had a 100% closure while the control animals had only a 85% closure as of day 20 POD.

The thickness of the epithelial layer in BP treated wounds was significantly higher in BP treated animals compared to the control animals, as shown in Table 1. The data represents the thickness of neodermis in mm measured on day 11 for the BP treated animals and day 16 for the control animals such that measurements are made at equivalent extents of healing. Histological analysis revealed that the wounds were closed by the human cells from the grafted material and there was collagen deposition in the closed wounds as revealed by involucrin and collagen type 1 immuno histological staining (data not shown). The capillary density in the wound bed following BP treatment was also significantly higher at the time of wound closure compared to untreated controls, as shown in Table 1. Further, in the animals treated with the lower BP dosage, there was a significant increase in the smooth muscle cell (SMC) count in the BP treated wounds as compared to the controls, as also seen in Table 1.

Table 1. Wound Thickness, Capillary Count and SMC Count for BP and Control Treated Wounds.

	Treatment		
	100 μg BP (n=5)	200 μg BP (n=5)	Control (n=10)
Epithelial Thickness (mm)	1.60 ± 0.12 (P<0.001)	1.55 ± 0.09 (P<0.001)	1.1 ±0.25
Capillary/Field	37 ±6 (P<0.01)	35 ± 7 (P<0.01)	25± 5.9
SMC counts/Field	53 ± 3.5 (P<0.001)	46.8 ± 4.4 (P<0.05)	46 ± 5.8

In summary, a single dose application of BP was effective in reducing the healing time of full thickness wound in nude mice grafted with human meshed split thickness skin. Additionally, the thickness of the neodermis and the density of the capillaries in the treated wounds were significantly higher compared to the control group of animals. In contrast, bFGF, also an angiogenic growth factor, was shown to have a deleterious effect on epithelialization when tested in a similar animal model. (Cooper, M.L. et al., 1991; Clinical and experimental approaches to dermal and epidermal repair: normal and chronic wounds, pp 429-442; Weilly-Liss, Inc.).

EXAMPLE 2. BP IN HYDROGEL

A small number of animals (n=3) were treated with BP solubilized in a hydrogel (carboxy-methyl cellulose) in the same animal model as described above. In this study, it was observed that the wounds (n=2) treated with BP in the hydrogel showed initiation of epithelialization as early as 5 days post operation compared to the wounds treated with BP solubilized in 1% povidone, which showed initiation of epithelialization only at 8 days post operation (data not shown). In both instances, the control animals that received the carrier alone did not show initiation of epithelialization until POD 8. Detailed histology is being carried out on the tissue samples to determine the thickness of the neodermis and the degree of angiogenesis in the wounds treated with the hydrogel formulation. However, wound closure data is presented in Table 2, below.

Table 2. Percent Wound Closure for BP and Control Treated Wounds.

		Percent Wound Closure (%)			
	Animal #	POD 5	POD 8	POD 11	POD 14
*Control (no BP)	1	0	50	70	70
Control (hydrogel, no BP, no salts)	2	25	70	70	100
BP & hydrogel, no salts	3	0	70	90	100
BP & hydrogel, no salts	4	25	80	90	90
BP & hydrogel, salts (some precipitate formed, probably due to buffering salts)	5	0	80	90	100

* The control animal had very thin and fragile skin at the time of biopsy compared to the animals, which received BP.

In summary, the results were very promising although preliminary, showing quicker wound closure in BP treated than control animals. Thus, more extensive experiments were undertaken to confirm the results, as described below.

EXAMPLE 3. COMPARATIVE STUDY BETWEEN REGRENEX(r) AND BP

REGRANEX(r) (PDGF-BB), the only approved growth factor product in the market for treating diabetic foot ulcers, showed complete healing in 50% of the patient population compared to the 35% placebo gel treatment that demonstrated complete healing after repeat application for about 20 weeks in diabetic patients (see REGRANEX(r) U.S. full prescribing information - package insert). Hence, a comparison of REGRANEX(r) (tm) versus BP was undertaken in a study similar to that described above. The results are presented in Tables 3 and 4.

Table 3. BP, Hydrogel (HG) and Regranex® Treated Wounds and Percent Wound Closure (%), Epithelial Thickness (mm) and Degree of Angiogenesis (# Estimated Capillaries per 20x Field).

		Percent (%) Wound Closure				Epi Thick (μm)	Angio (#est cap/hpf 20x)
Animal #	Treatment Group	POD 5	POD 8	POD 11	POD 14	POD 14	POD 14
1	BP	10	25	85	100	17.5	28
2	BP	10					
3	BP	15					
4	BP	10					
5	BP	10	30	85	80	7.5	16
6	BP	10					
7	BP	10	10				
8	BP	10	30	85	100	11.5	26
9	BP	30	50	85	100	16	21
10	BP	30	50	85	100	12	20
11	BP	20	45	85	100	18	18
12	BP	10	15	85	90	6	20
13	BP	10	20	95	100	5.5	23
14	BP	15	25	90	100	10	32
15	BP	5	50	90	95	14	25
n		**15**	**11**	**10**	**10**	**10**	**10**
mean		**13.67**	**31.82**	**87.00**	**96.50**	**11.80**	**22.9**
SD		**7.43**	**14.71**	**3.50**	**6.69**	**4.58**	**4.88**
SEM		**0.54**	**0.46**	**0.04**	**0.07**	**0.39**	
16	HG	15	35	75	55	12.5	28
17	HG	10	60	70	95	10.5	5
18	HG	5	25	60	95	9	34
19	HG	10	30	70	90	17.5	8
20	HG	20	40	80	95	17.5	20
21	HG	10	10	80	95	13	15
22	HG	30	80	70	90	10	
23	HG	10	80	80	90	20	10
24	HG	15	40	70	90	18	15
25	HG	20	35	70	90	10.5	16
26	HG	10	10	70	90	12.5	20
27	HG	10	35	70	90	8	32
28	HG	10	55				
29	HG	5	40				
30	HG	15	40	70			
n		**15**	**15**	**13**	**12**	**12**	**11**
mean		**13.00**	**41.00**	**71.92**	**88.75**	**13.25**	**18.455**

SD		**6.49**	**20.72**	**5.60**	**10.90**	**4.01**	**9.55**
SEM		**0.50**	**0.51**	**0.08**	**0.12**	**0.30**	
31	Regranex	20	30	55	75	16	
32	Regranex	15	80				13
33	Regranex	20	80	100	100	8.5	4
34	Regranex	15	50	90	100	10	
35	Regranex	40	75				6
36	Regranex	15	70	90	100	7.5	10
37	Regranex	15	70	90		18	
38	Regranex	10	80				
39	Regranex	40	80				
40	Regranex	15	50	80	90	15	13
41	Regranex	15	10				
42	Regranex	5	50	100	100	16	21
43	Regranex	40	70	100	100	22.5	10
44	Regranex	5	40	80	100	16.5	6
45	Regranex						
n		**14**	**14**	**9**	**8**	**9**	**9**
mean		**19.29**	**59.64**	**87.22**	**95.63**	**14.44**	**10.375**
SD		**12.07**	**21.88**	**14.39**	**9.04**	**4.88**	**5.4**
SEM		**0.63**	**0.37**	**0.16**	**0.09**	**0.34**	

The percent closure results can be summarized as follows:

Table 4. Summary

	POD's	BP (mean)	HG (mean)	REG (mean)
wound closure (%)	0	0.00	0.00	0.00
	5	13.67	13.00	19.29
	8	31.82	41.33	59.64
	11	87.00	71.92	87.22
	14	96.25	89.17	95.63
epithelial thickness (mm)	14	11.8	13.25	14.44
angiogenesis (#/filed)	14	22.9	18.45	10.38

Thus, the BP treatment is as good as REGRENEX(tm) in closing wounds although slightly slower healing rates are initially observed. BP treatment also shows slightly less thickening of the epithelium and shows considerably improved angiogenesis in the wound area.

EXAMPLE 4. FUTURE APPLICATIONS

Because BP has shown promise as a wound healing agent, it will next be tested in applications where wound healing is known to be deficient. Experiments similar to those described above will be performed with diabetic animals to test the healing of full and partial thickness wounds. The response of venous stasis ulcers and diabetic ulcers to BP will also be tested.

In preliminary experiments, Male Sprague Dawley rats weighing greater than 325 g were rendered diabetic by treatment with streptozotocin and the hyperglycemia was confirmed by glucometry. Four full thickness incisional wounds were introduced on the dorsal surface of each animal perpendicular to the longitudinal axis. The wounds were closed with silk sutures and the growth factor or the placebo applied into the wound gap or on top of the incision after closure. The application was done at two time points: 1) on day 0, which is on the day of introducing the wound (surgery) and a second application 2) on day 3 following the introduction of the wound. The incisional strength was measured on day 7 and day 10 after surgery. The data is given in Table 5 and is very encouraging that the BP treatment will be particularly useful in treating a variety of diabetic ulcers, or other wounds characterized by delayed and/or poor healing.

Table 5. Tensile Strength of Wounds in Diabetic Rats

	Tensile Strength (kg/mm) ± sem	
	Control	BP
Day 7	3.6 ± 1	4.2 ± .7
Day 10	5.2 ± .7	9.1 ± .8

EXAMPLE 5: FURTHER CHARACTERIZATION OF BP

The BP has been partially characterized as follows: high performance liquid chromatography ("HPLC") fractions have been denatured, reduced with DTT, and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). One minute HPLC fractions from 27 to 36 minutes are shown in Figure 2. Size standards (ST) of 14, 21, 31, 45, 68 and 97 kDa were obtained as Low Range size standards from BIORAD(tm) and are shown at either end of the coomassie blue stained gel. In the usual protocol, HPLC fractions 29 through 34 are pooled to produce BP (see boxes, Figures 2 and 3), as shown in a similarly prepared SDS-PAGE gel in Figure 17B.

The various components of the BP were characterized by mass spectrometry and amino acid sequencing of tryptic fragments where there were sufficient levels of protein for

analysis. The major bands in the 1D gel (as numerically identified in Figure 3) were excised, eluted, subjected to tryptic digestion and the fragments were HPLC purified and sequenced. The sequence data was compared against known sequences, and the best matches are shown in Figures 15A-B. These identifications are somewhat tentative in that only portions of the entire proteins have been sequenced and, in some cases, there is variation between the human and bovine analogs for a given protein.

The same tryptic protein fragments were analyzed by mass spectrometry and the mass spectrograms are shown in Figures 7A-O. The tabulated results and homologies are shown in Figures 16A-F which provides identification information for the bands identified in Figures 3-4. As above, assignment of spot identity may be tentative based on species differences and post-translational modifications. A summary of all protein identifications from ID gels is shown in Figure 4.

The identified protein components of BP, as described in Figures 15A-B, 16A-F and 19A-D, were quantified as shown in Figures 17A and 17B. Figure 17B is a stained SDS-PAGE gel of BP and Figure 17A represents a scanning densitometer trace of the same gel. The identified proteins were labeled and quantified by measuring the area under the curve. These results are presented in Figure 18 as a percentage of the total peak area.

Thus, there are 11 major bands in the BP SDS-PAGE gel representing about 60% of the protein in BP. The identified proteins fall roughly into three categories: the ribosomal proteins, the histones and growth factors, including bone morphogenic factors (BMPs). It is expected that the ribosomal proteins and histone proteins may be removed from the BP without loss of activity, since these proteins are known to have no growth factor activity. Upon this separation, the specific activity is expected to increase correspondingly.

Experiments are planned to confirm the hypothesis that the histone and ribosomal proteins may be removed from the BP with no resulting loss, or even an increase, in specific activity. Histones will be removed from the BP cocktail by immunoaffinity chromatography using either specific histone protein antibodies or a pan-histone antibody. The histone depleted BP (BP-H) will be tested as described above for wound healing and/or osteogenic activity. Similarly, the known ribosomal proteins will be stripped and the remaining mixture (BP-R) tested.

An SDS-PAGE gel of BP was also analyzed by Western immunoblot with a series of antibodies, as listed in Figure 14. Visualization of antibody reactivity was by horse radish peroxidase conjugated to a second antibody and using a chemiluminescent substrate. Further, TGF-ß1 was quantified using commercially pure TGF-ß1 as a standard and was

determined to represent less than 1% of the BP protein. The antibody analysis indicated that each of the proteins listed in Figure 14 is present in BP.

The BP was further characterized by 2-D gel electrophoresis, as shown in Figures 5-6. The proteins are separated in horizontal direction according to charge (pI) and in the vertical direction by size as described in two-dimensional electrophoresis adapted for resolution of basic proteins was performed according to the method of O'Farrell et al. (O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H., Cell, 12: 1133-1142, 1977) by the Kendrick Laboratory (Madison, WI). Two-dimensional gel electrophoresis techniques are known to those of skill in the art. Nonequilibrium pH gradient electrophoresis ("NEPHGE") using 1.5% pH 3.5-10, and 0.25% pH 9-11 ampholines (Amersham Pharmacia Biotech, Piscataway, NJ) was carried out at 200 V for 12 hrs. Purified tropomyosin (lower spot, 33,000 KDa, pI 5.2), and purified lysozyme (14,000 KDa, pI 10.5 - 11) (Merck Index) were added to the samples as internal pI markers and are marked with arrows.

After equilibration for 10 min in buffer "0" (10% glycerol, 50 mM dithiothreitol, 2.3% SDS and 0.0625 M tris, pH 6.8) the tube gel was sealed to the top of a stacking gel which is on top of a 12.5% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 4 hrs at 12.5 mA/gel.

After slab gel electrophoresis two of the gels were coomassie blue stained and the other two were transferred to transfer buffer (12.5 mM Tris, pH 8.8, 86 mM Glycine, 10% MeoH) transblotted onto PVDF paper overnight at 200 mA and approximately 100 volts/two gels. The following proteins (Sigma Chemical Co., St. Louis, MO) were added as molecular weight standards to the agarose which sealed the tube gel to the slab gel: myosin (220,000 KDa), phosphorylase A (94,000 KDa), catalase (60,000 KDa), actin (43,000 KDa), carbonic anhydrase (29,000 KDa) and lysozyme (14,000 KDa). Figure 5 shows the stained 2-D gel with size standards indicated on the left. Tropomyosin (left arrow) and lysozyme (right arrow) are also indicated.

The same gel is shown in Figure 6 with several identified proteins indicated by numbered circles. The proteins were identified by mass spectrometry and amino acid sequencing of tryptic peptides, as described above. The identity of each of the labeled circles is provided in the legend of Figure 6 and the data identifying the various protein spots is presented in Figures 19A-D.

Because several of the proteins migrated at more than one size (e.g., BMP-3 migrating as 6 bands) investigations were undertaken to investigate the extent of post-translation

modification of the BP components. Phosphorylation was measured by anti-phosphotyrosine immunoblot and by phosphatase studies. Figure 8 shows a 2-D gel, electroblotted onto filter paper and probed with a phosphotyrosine mouse monoclonal antibody by SIGMA (# A-5964). Several proteins were thus shown to be phosphorylated at one or more tyrosine residues.

Similar 2-D electroblots were probed with BP component specific antibodies, as shown in Figures 9A-D. The filters were probed with BMP-2, BMP-3 (Fig. 9A), BMP-3, BMP-7 (Fig. 9B), BMP-7, BMP-2 (Fig. 9C), and BMP-3 and TGF-ß1 (Fig. 9D). Each shows the characteristic, single-size band migrating at varying pI, as is typical of a protein existing in various phosphorylation states.

For the phosphatase studies, BP in 10 mM HCl was incubated overnight at 37° C with 0.4 units of acid phosphatase (AcP). Treated and untreated samples were added to lyophilized discs of type I collagen and evaluated side by side in the subcutaneous implant rat bioassay, as previously described in U.S. Patent Nos. 5,290,763, 5,563,124 and 5,371,191. Briefly, 10 (g of BP in solution was added to lyophilized collagen discs and the discs implanted subcutaneously in the chest of a rat. The discs were then recovered from the rat at 2 weeks for the alkaline phosphotase ("ALP" - a marker for bone and cartilage producing cells) assay or at 3 weeks for histological analysis. For ALP analysis of the samples, the explants were homogenized and levels of ALP activity measured using a commercial kit. For histology, thin sections of the explant were cut with a microtome, and the sections stained and analyzed for bone and cartilage formation.

Both native- and phosphatase-treated BP samples were assayed for morphogenic activity by mass of the subcutaneous implant (explant mass) and ALP score. The results showed that AcP treatment reduced the explant mass and ALP score from 100% to about 60%. Thus, phosphorylation is important for BP activity.

The BP was also analyzed for glycosylation. Figure 10 shows an SDS-PAGE gel stained with periodic acid schiff (PAS) - a non-specific carbohydrate stain, indicating that several of the BP components are glycosylated (starred protein identified as BMP-3). Figures 11-12 show immunodetection of two specific proteins (BMP-7, Fig. 11 and BMP-2, Fig. 12) treated with increasing levels of PNGase F (Peptide-N-Glycosidase F). Both BMP-2 and BMP-7 show some degree of glycoslyation in BP, but appear to have some level of protein resistant to PNGase F as well (plus signs indicate increasing levels of enzyme). Functional activity of PNGase F and sialadase treated samples were assayed by explant

mass and by ALP score, as shown in Figure 13A and 13B which shows that glycosylation is required for full activity.

In summary, BMPs 2, 3 and 7 are modified by phosphorylation and glycosylation. These post-translation modifications affect protein morphogenic activity, 33% and 50% respectively, and care must be taken in preparing BP not to degrade these functional derivatives.

WHAT IS CLAIMED IS:

1. A composition for the treatment of wounds, said composition comprising the growth factors BMP-3 and TGF-ß2 in a pharmaceutically acceptable carrier.

2. The composition of claim 1, further comprising a growth factor selected from the group consisting of BMP-2, BMP-4, BMP-5, BMP-6, and BMP-7.

3. The composition of claim 2, further comprising a growth factor selected from the group consisting of FGF-1, TGF-ß1, and TGF-ß3.

4. The composition of claim 3, wherein the growth factors are derived from a natural source and are at least partially phosphorylated and glycosylated.

5. The composition of claim 1, excluding histone proteins H1c and H1x.

6. A composition for the treatment of wounds, said composition comprising a mixture of growth factors comprising BMP-2, BMP-3, BMP-6, and TGF-ß2 in a pharmaceutically acceptable carrier.

7. The composition of claim 6, from which ribosomal proteins LORP, L6, S20, L3, S3a, S4 and L32 have been substantially excluded.

8. The composition of claim 7, wherein the growth factors are derived from bovine bone and are at least partially phosphorylated and glycosylated.

9. A composition for the treatment of wounds, said composition comprising a mixture of proteins as identified in Figure 1, wherein the histone proteins have been excluded from the mixture, said mixture being in a pharmaceutically acceptable carrier.

10. The composition of claim 9, wherein the ribosomal proteins have been excluded therefrom.

11. A composition for the treatment of wounds, said composition comprising a mixture of proteins components as identified in Figure 1, wherein the ribosomal proteins have been excluded therefrom, said components being in a pharmaceutically acceptable carrier.

12. The composition of claim 11, wherein the histone proteins have been excluded therefrom.

13. A composition for the treatment of wounds, said composition comprising a mixture of proteins comprising BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, TGF-ß1, TGF-ß2, and TGF-ß2, and FGF-1 in a pharmaceutically acceptable carrier.

14. The composition of claim 13, wherein ribosomal proteins have been substantially eliminated from the mixture.

15. The composition of claim 13, wherein histone proteins have been substantially eliminated from the mixture.

16. The composition claim 13, wherein the components are isolated from a natural source and are at least partially phosphorylated and glycosylated.

17. The composition of claim 13, wherein at least one of the components is a recombinantly produced protein.

18. A method of wound healing, said method comprising applying a composition as in claims 13 to a wound.

19. The method of claim 18, where the pharmaceutically acceptable carrier includes a hydrogel.

20. The method of claim 18, wherein the components are isolated from a natural source and are at least partially phosphorylated and glycosylated.

21. The method of claim 18, where the pharmaceutically acceptable carrier includes a dressing selected from the group consisting of hydrocolloid dressings, hydrogels, foam dressings, and alginate dressings.

22. The method of claim 18, further including one or more active ingredient selected from the group consisting of arginine, glutamine, zinc, copper, vitamin C, vitamin B1, vitamin B2, vitamin B3, vitamin B6, vitamin B12, and folate.

23. The method of claim 18, further including one or more growth factor selected from the group consisting of epidermal growth factor, platelet derived growth factor, insulin-like growth factor, keratinocyte growth factor, vascular endothelial growth factor, transforming growth factor alpha, nerve growth factor, connective tissue growth factor and granulocyte-monocyte colony stimulating factor.

24. The method of claim 11, further including one or more inflammation inhibitor selected from the group consisting of interleukin-1 inhibitor, interleukin-6 inhibitor and tumor necrosis factor-alpha inhibitor.

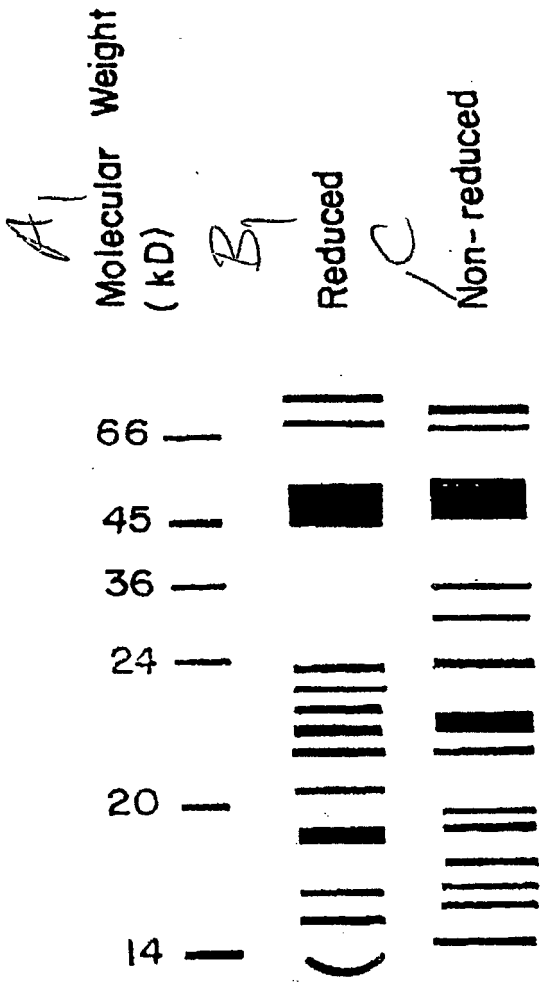
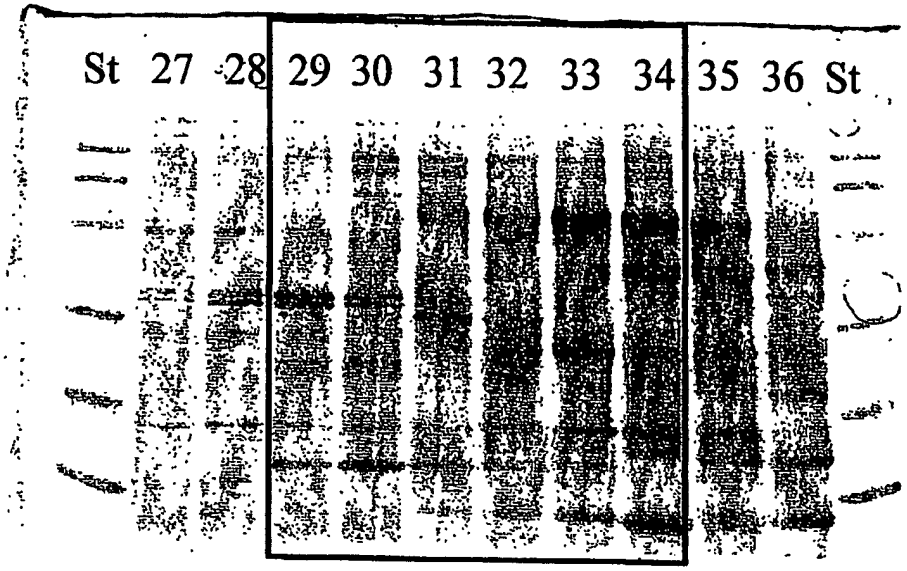


FIG. 1

FIGURE 2



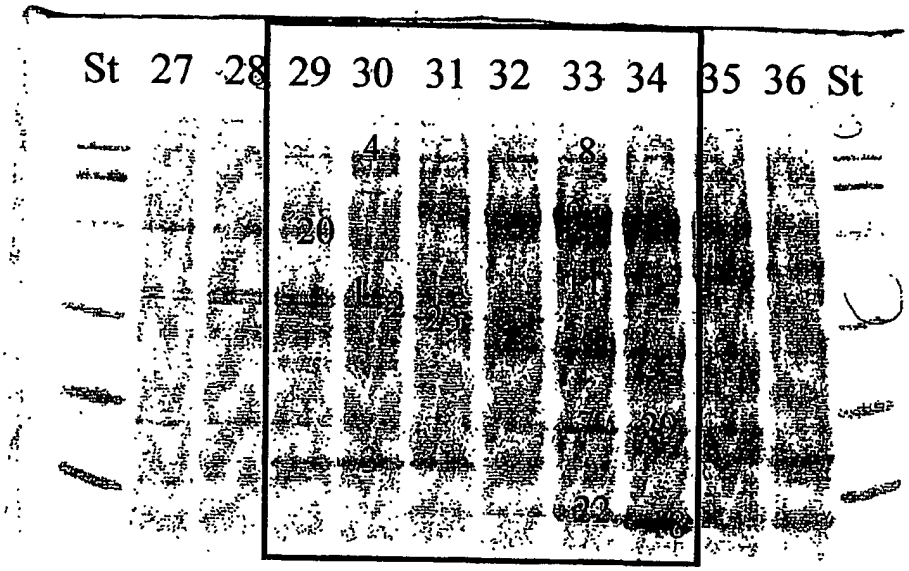
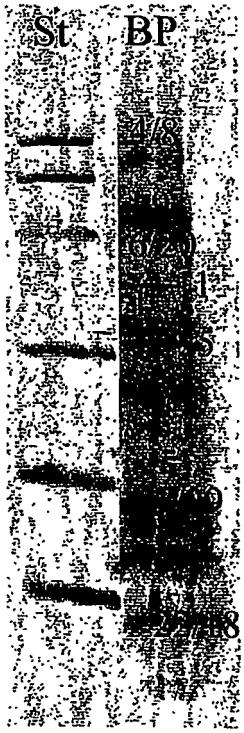


FIGURE 3



Band No.	Identity
1	Histone H1.c
2	Histone H1.c
3	Ribosomal protein RS20
4	Similar to ribosomal protein LORP
5	BMP-3
6	α2 macroglobulin RAP and BMP-3
7	Similar to ribosomal protein LORP
8	BMP-3
9	BMP-3
11	Ribosomal protein RL6 and BMP-3
18	TGF-β2 / SPP 24
20	Factor H
22	TGF-β2
25	BMP-3 and H1.x
29	BMP-3 and ribosomal protein RL32

FIGURE 4

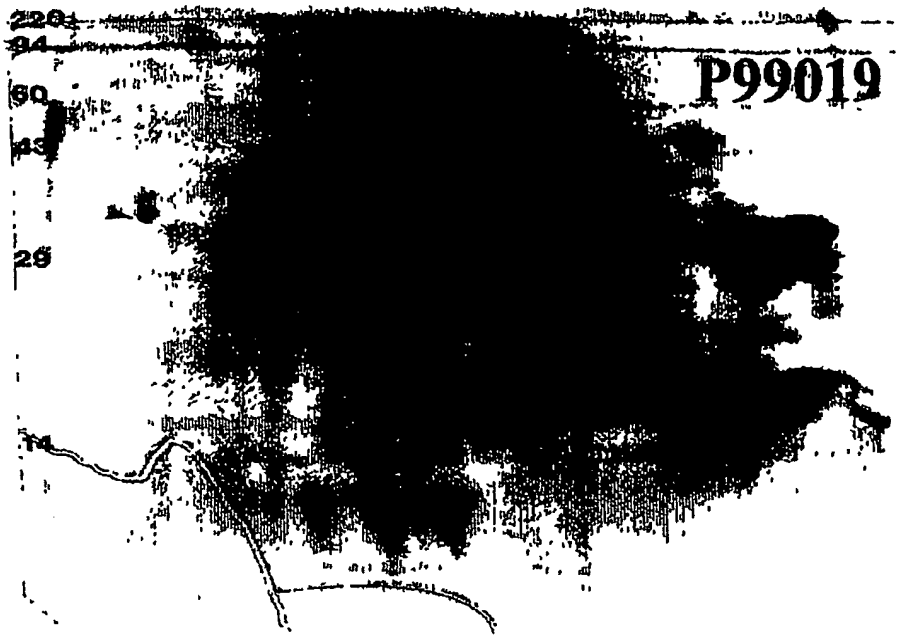


FIGURE 5

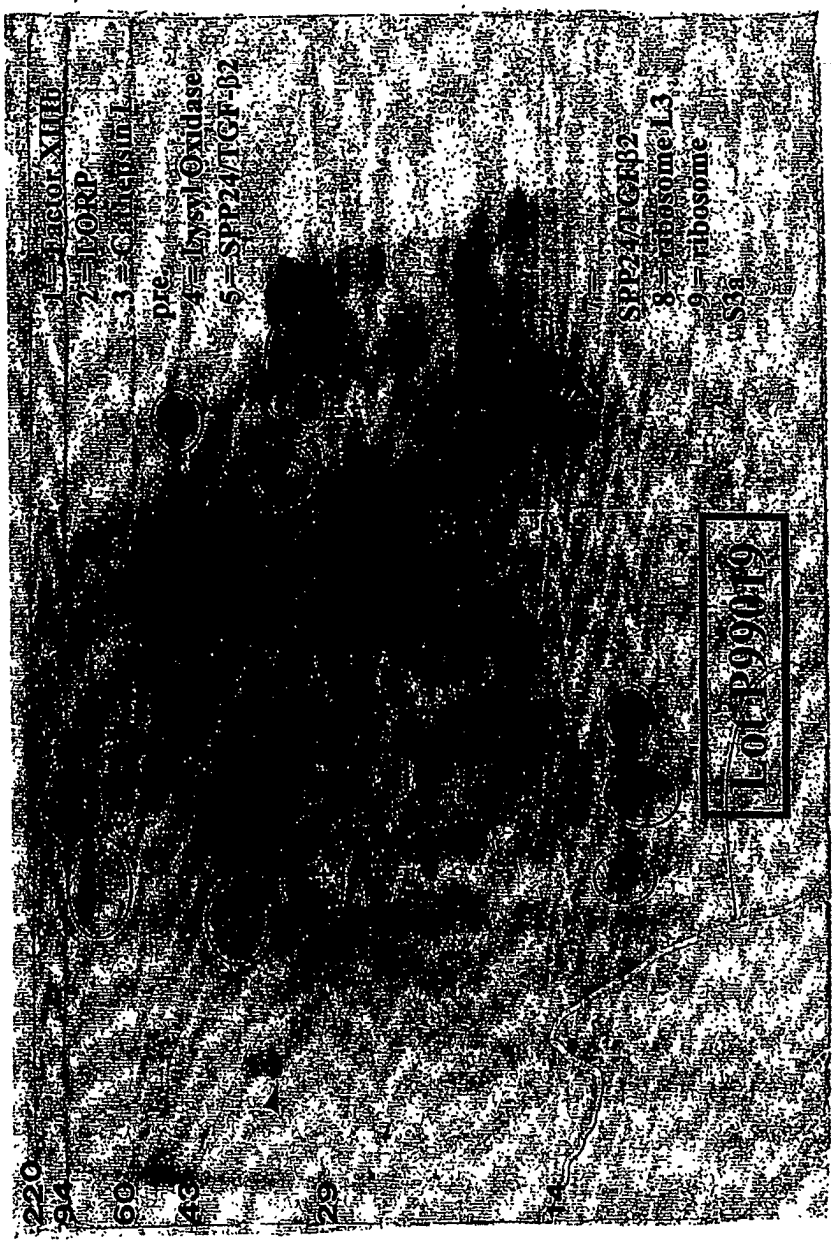

1 = Factor XIIIb
2 = LORP
3 = Calreticulin
pre
4 = Lysyl Oxidase
5 = SPP24/TGF-β2
SPP24/TGFβ2
3 = ribosome L3
9 = ribosome
uS3a
L01P99013
220
294
60
43
29
44

FIGURE 6

Figure 7A (Band 1)

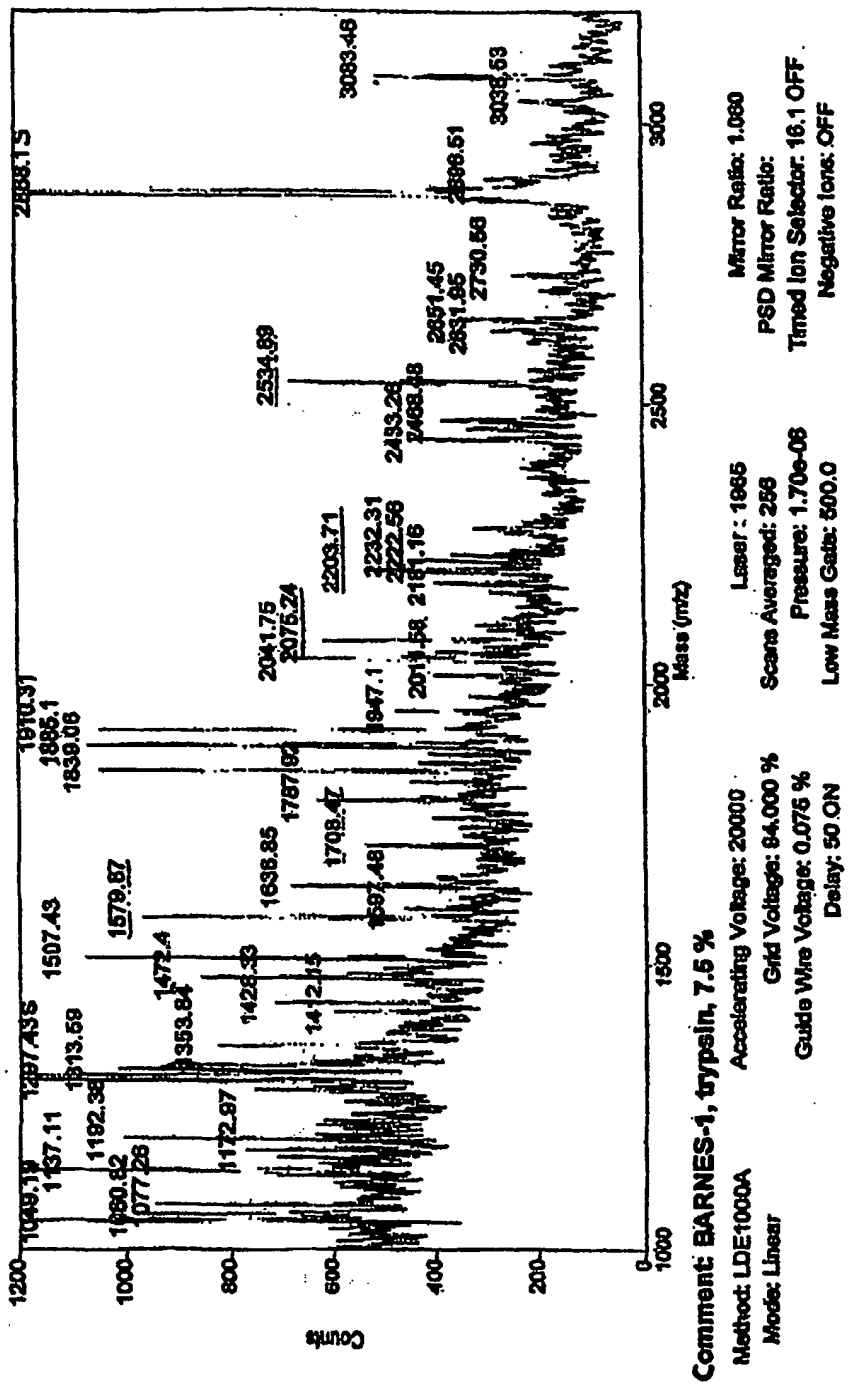


Figure 7B (Band 2)

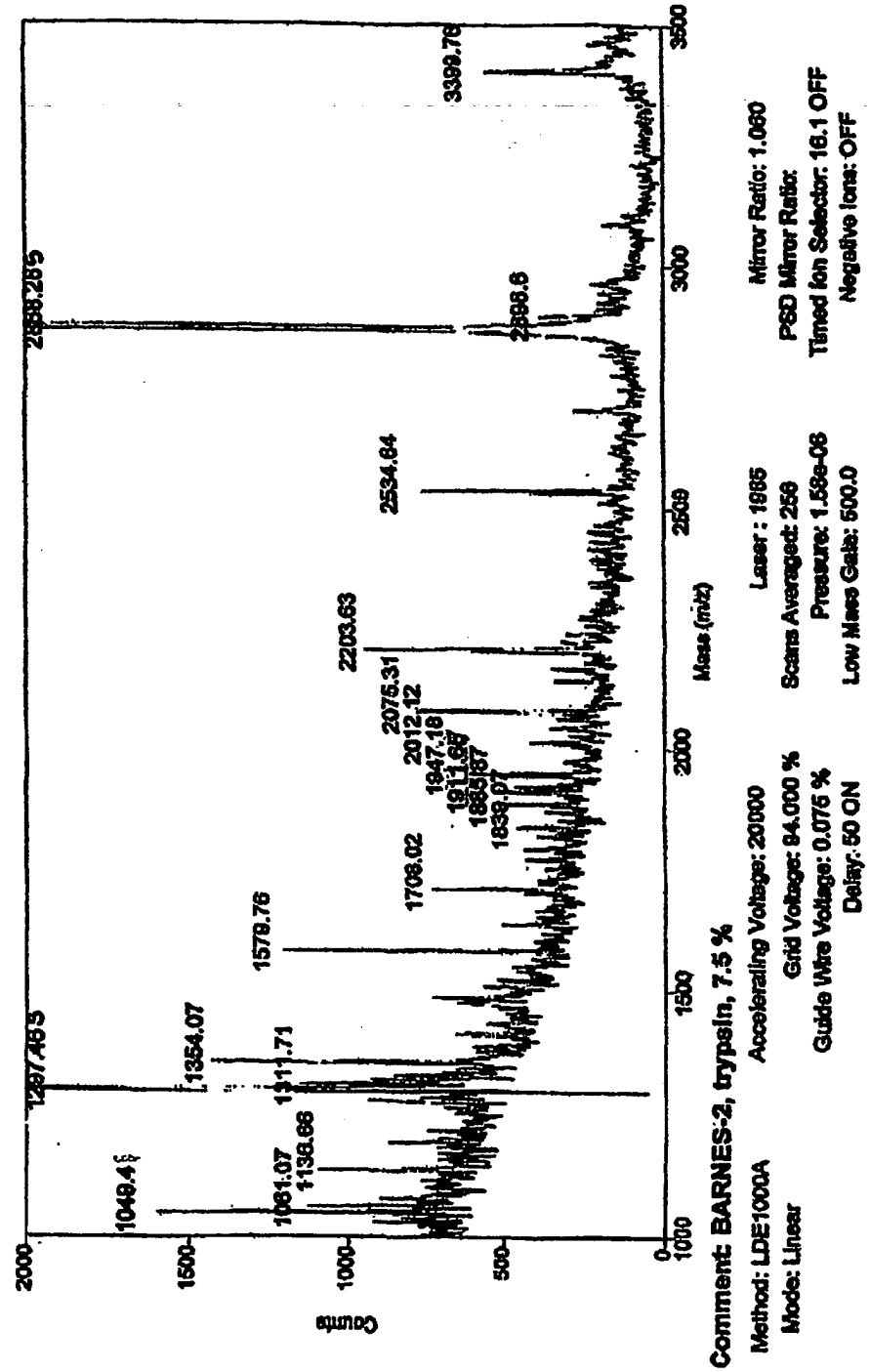


Figure 7C (Band 3)

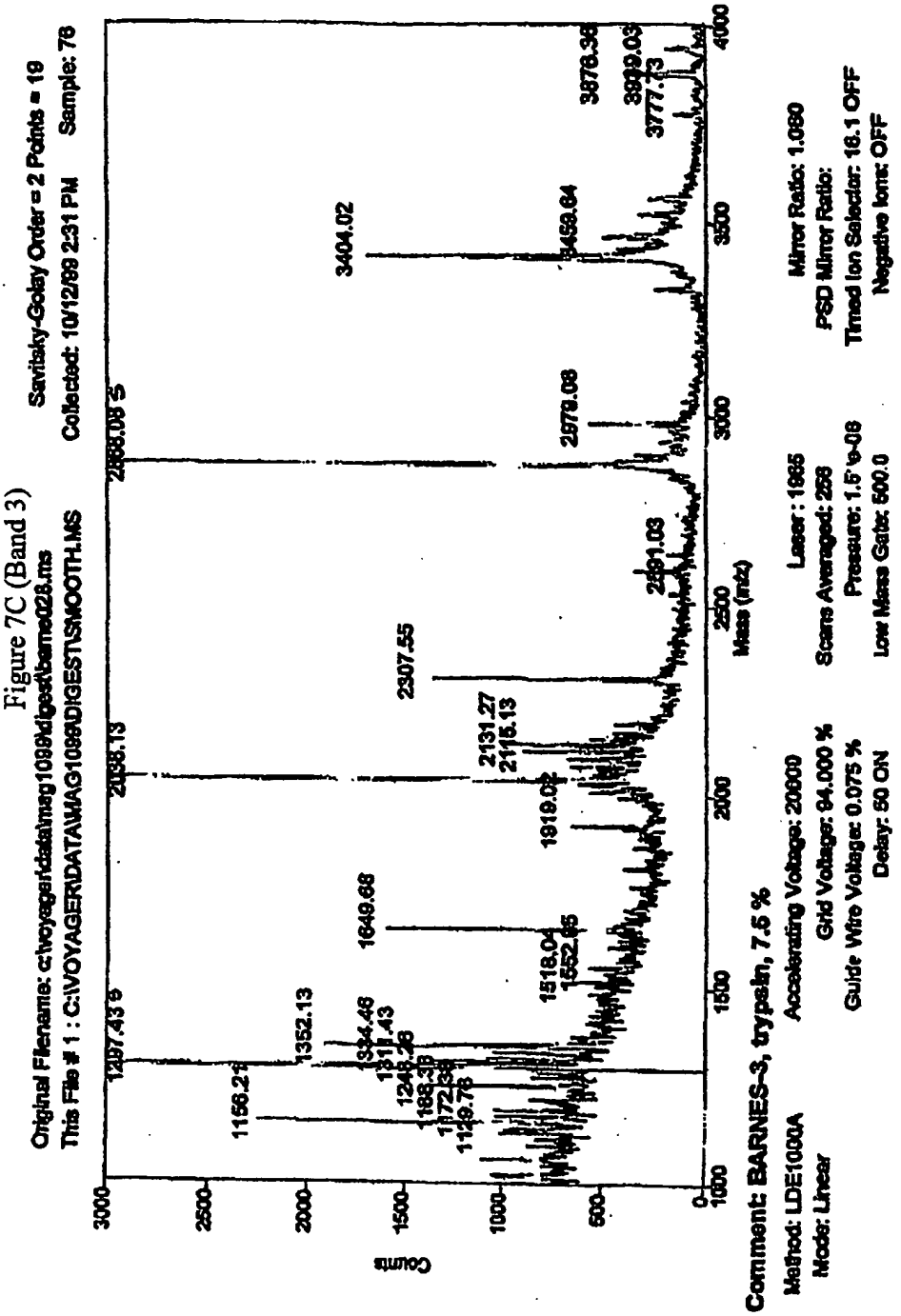


Figure 7D (Band 4)

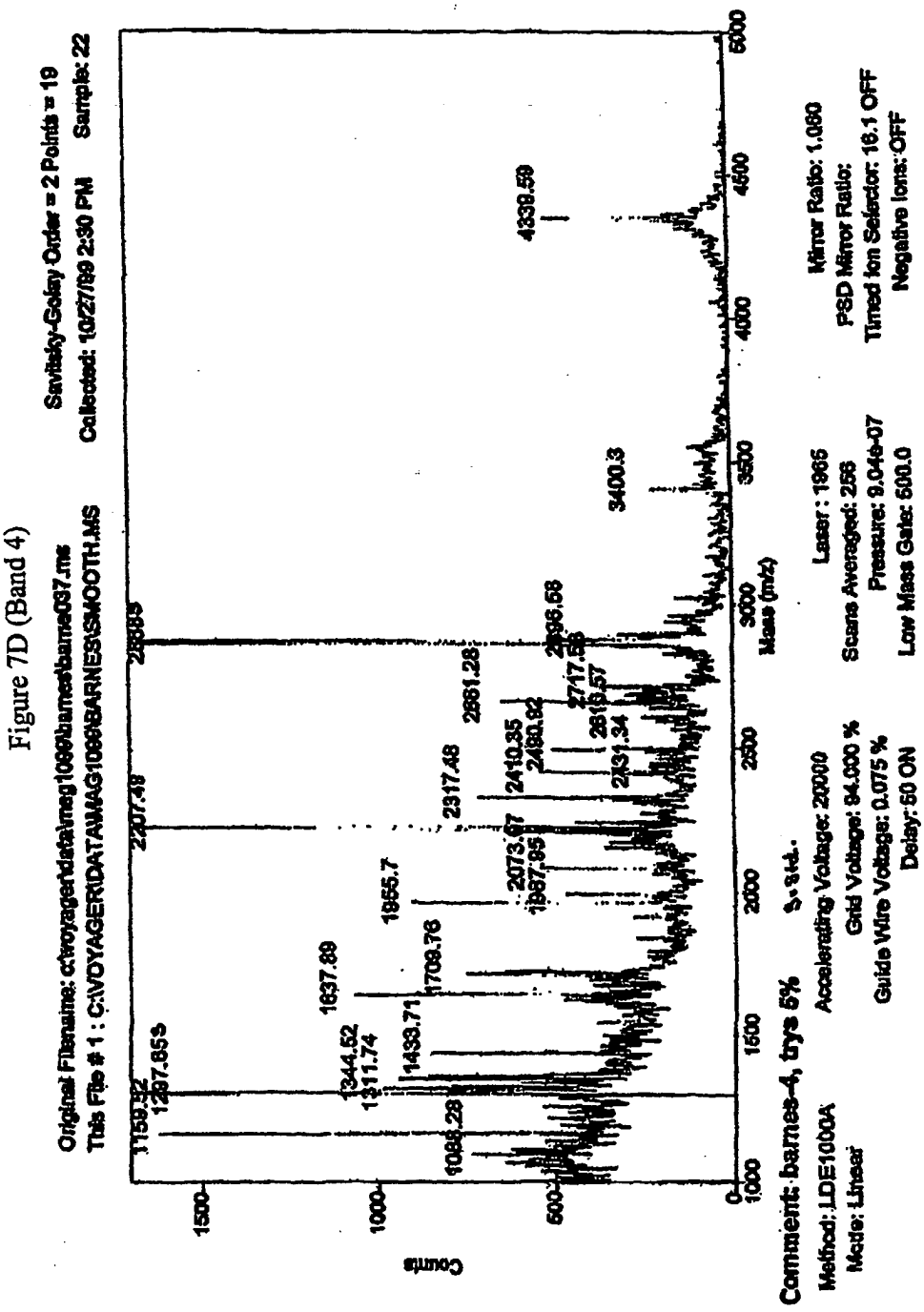


Figure 7E (Band 5)

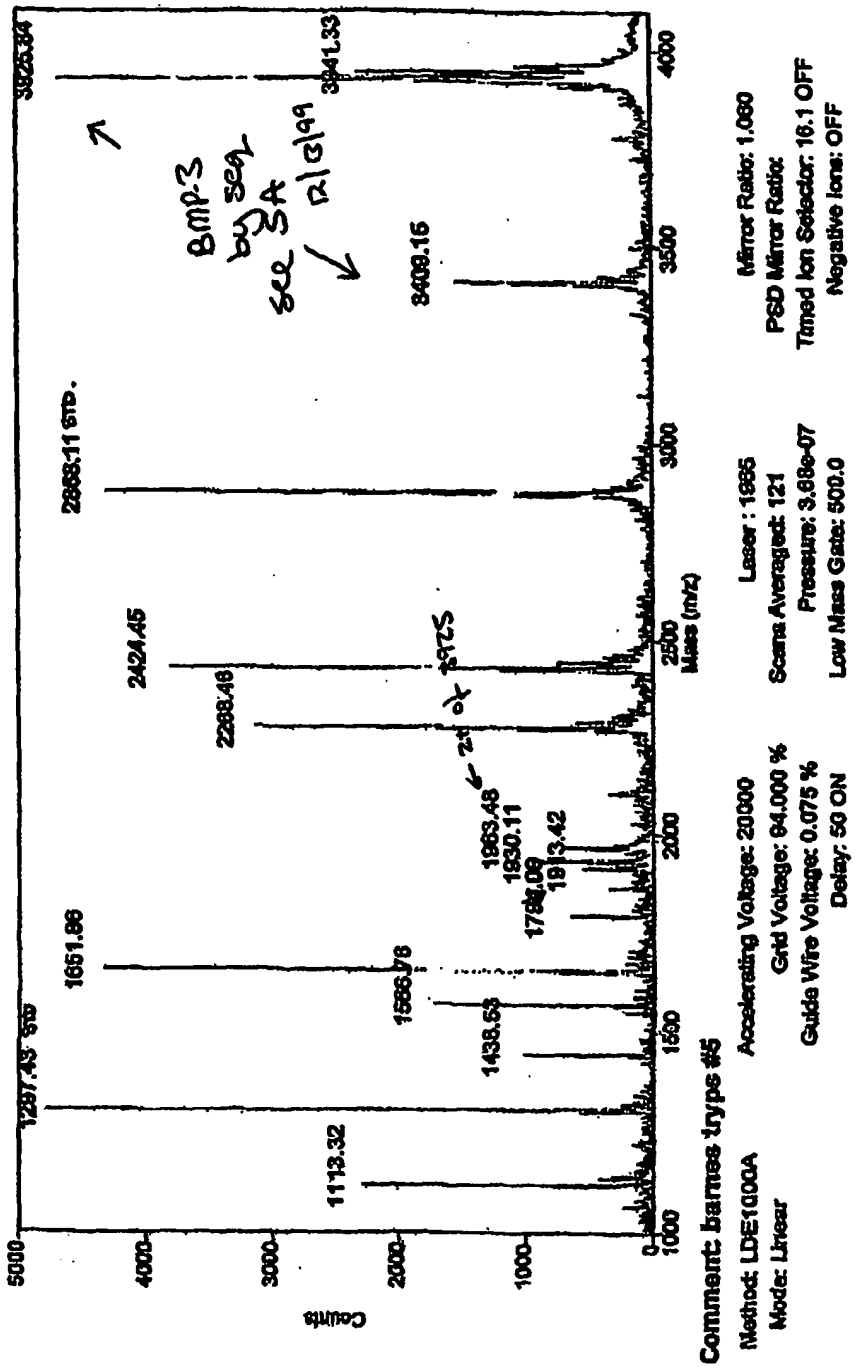


Figure 7F (Band 6)

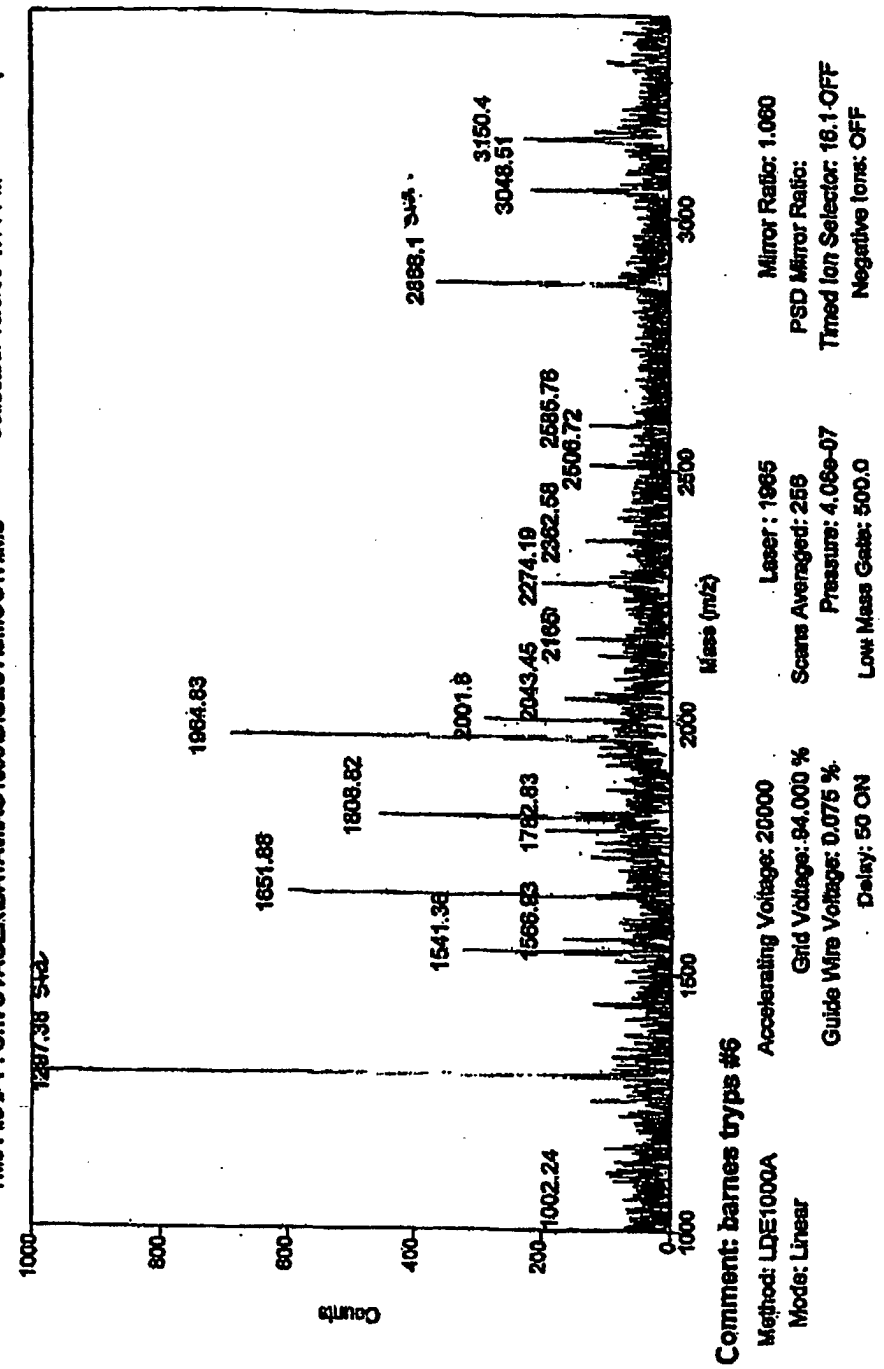


Figure 7G (Band 7)

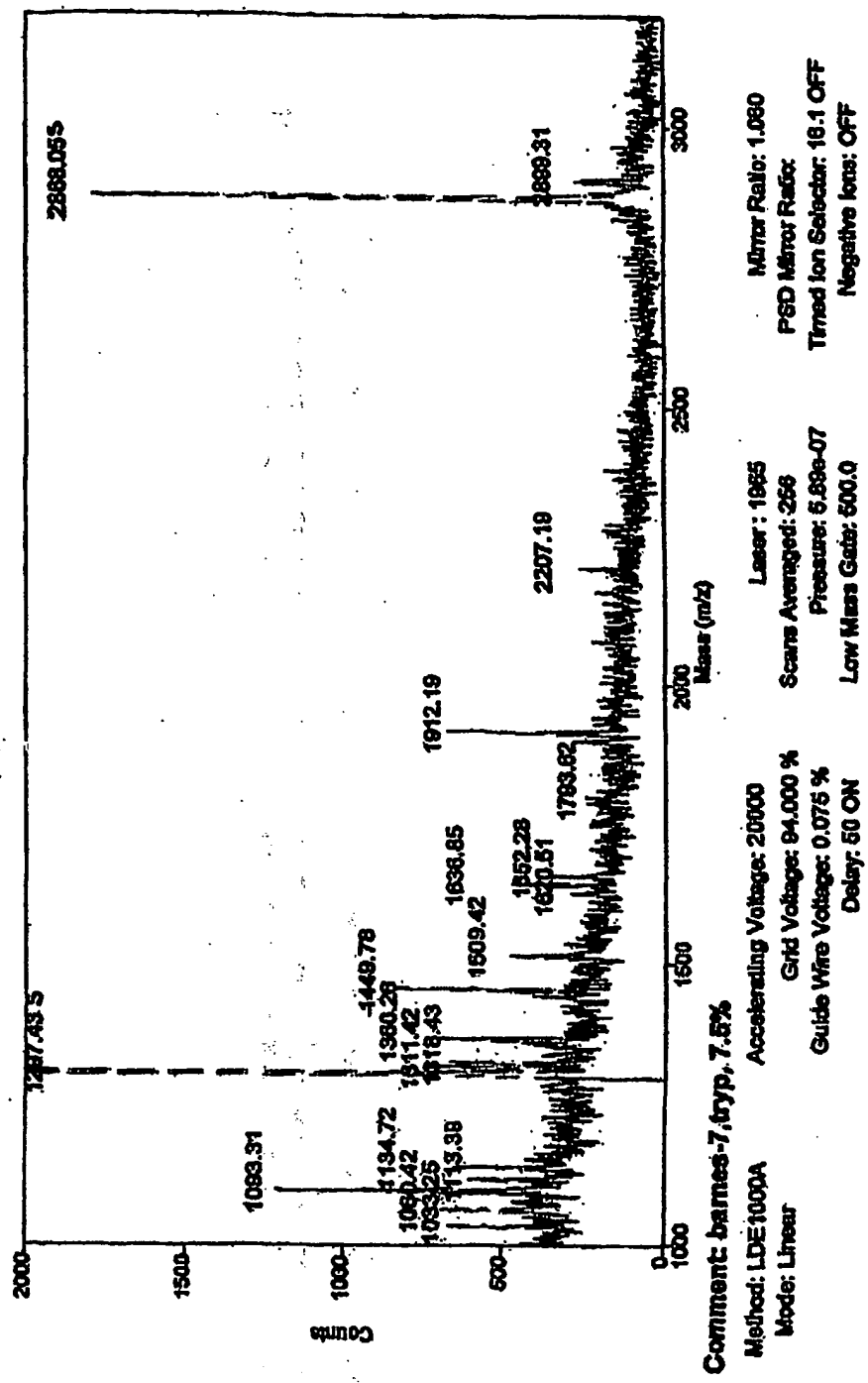


Figure 7H (Band 8)

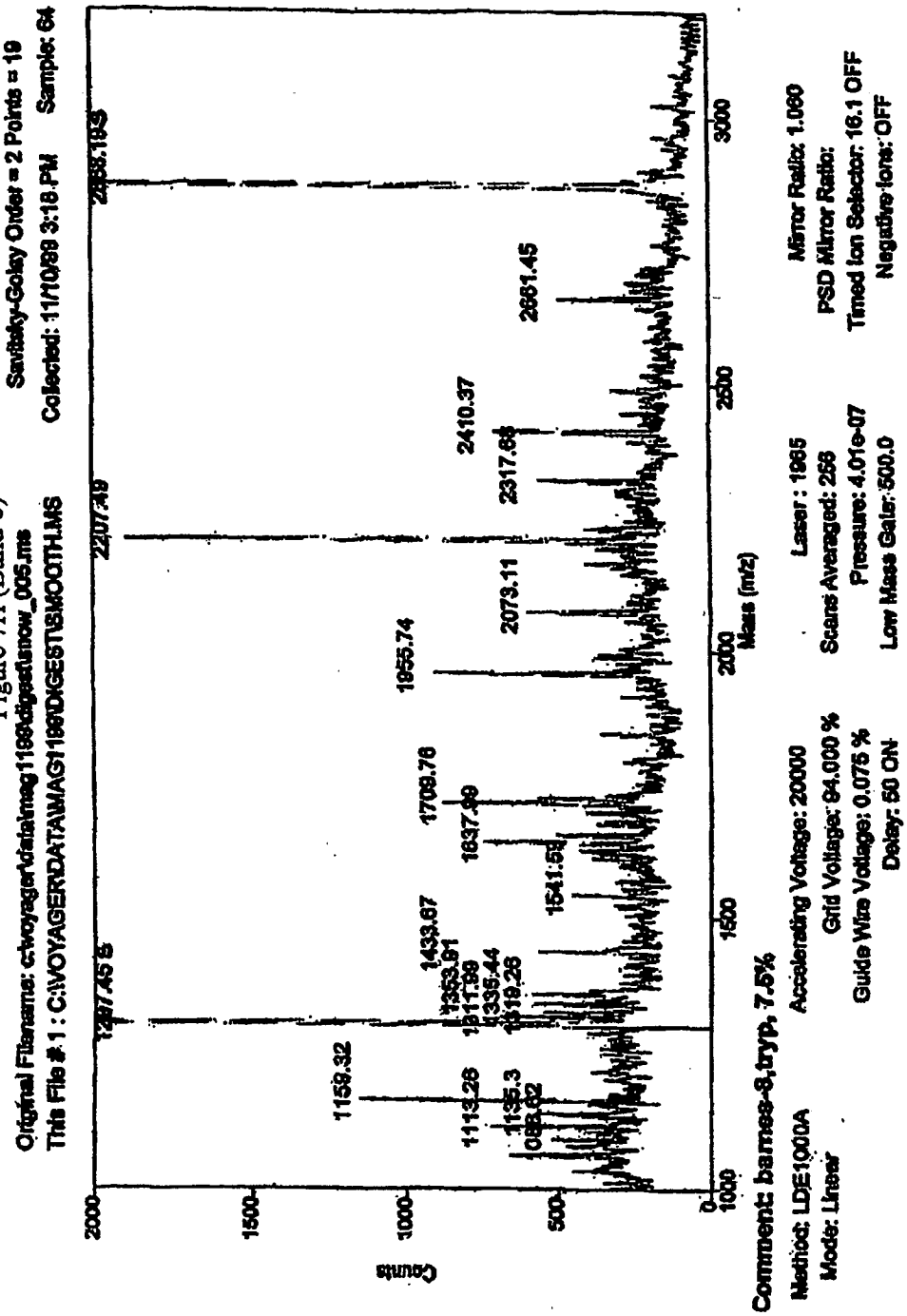


Figure 7I (Band 9)

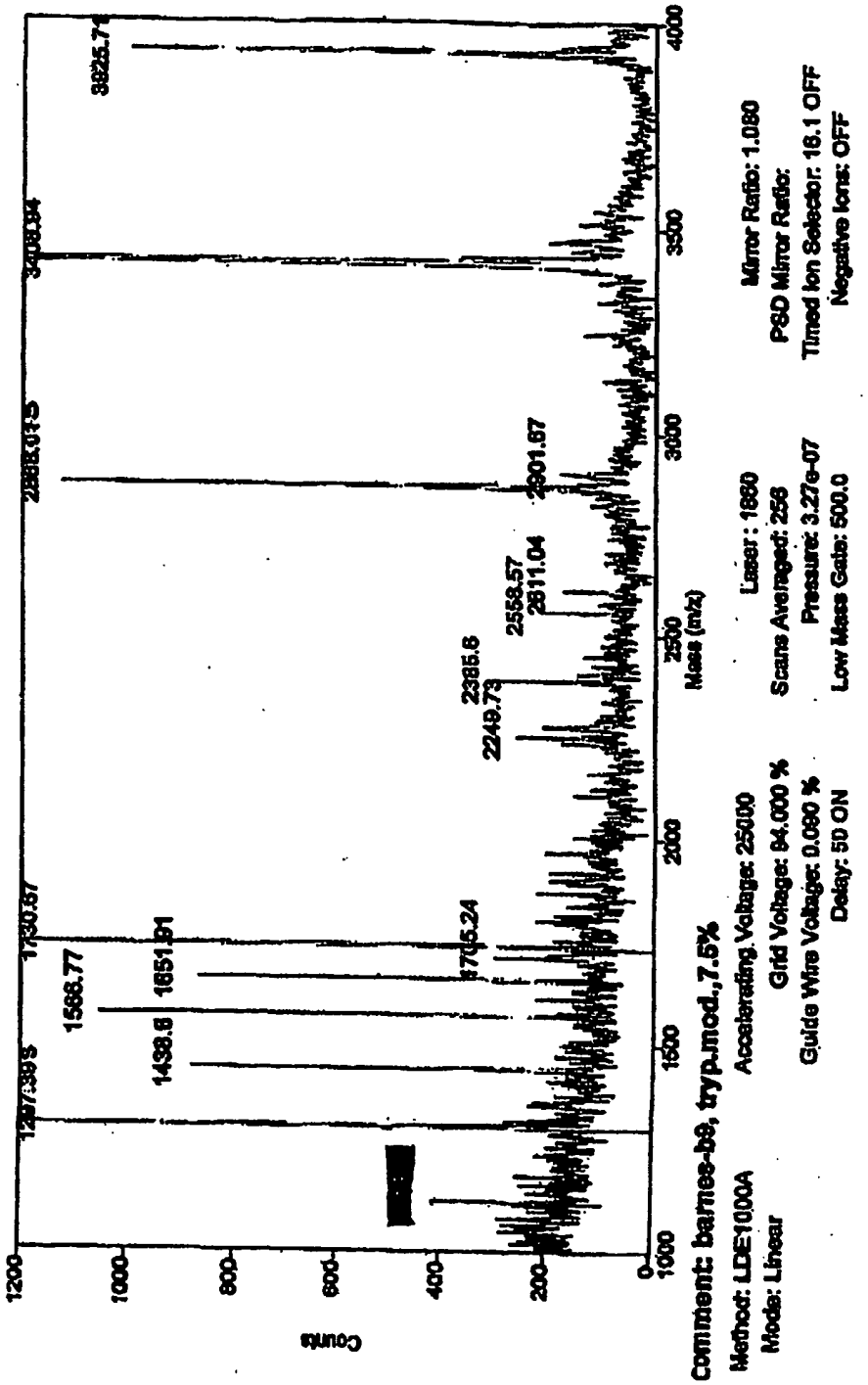


Figure 7J (Band 11)

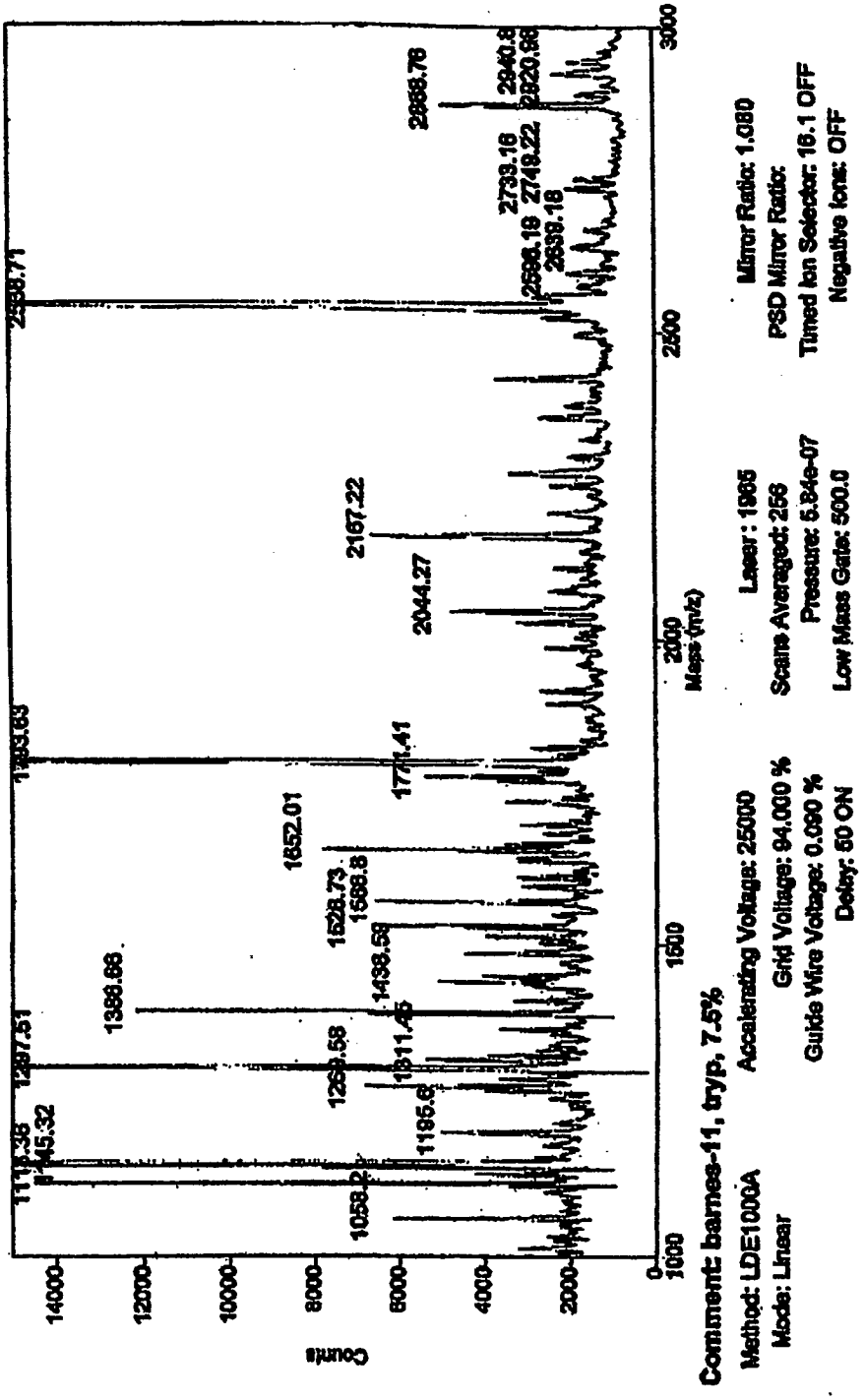


Figure 7K (Band 18)

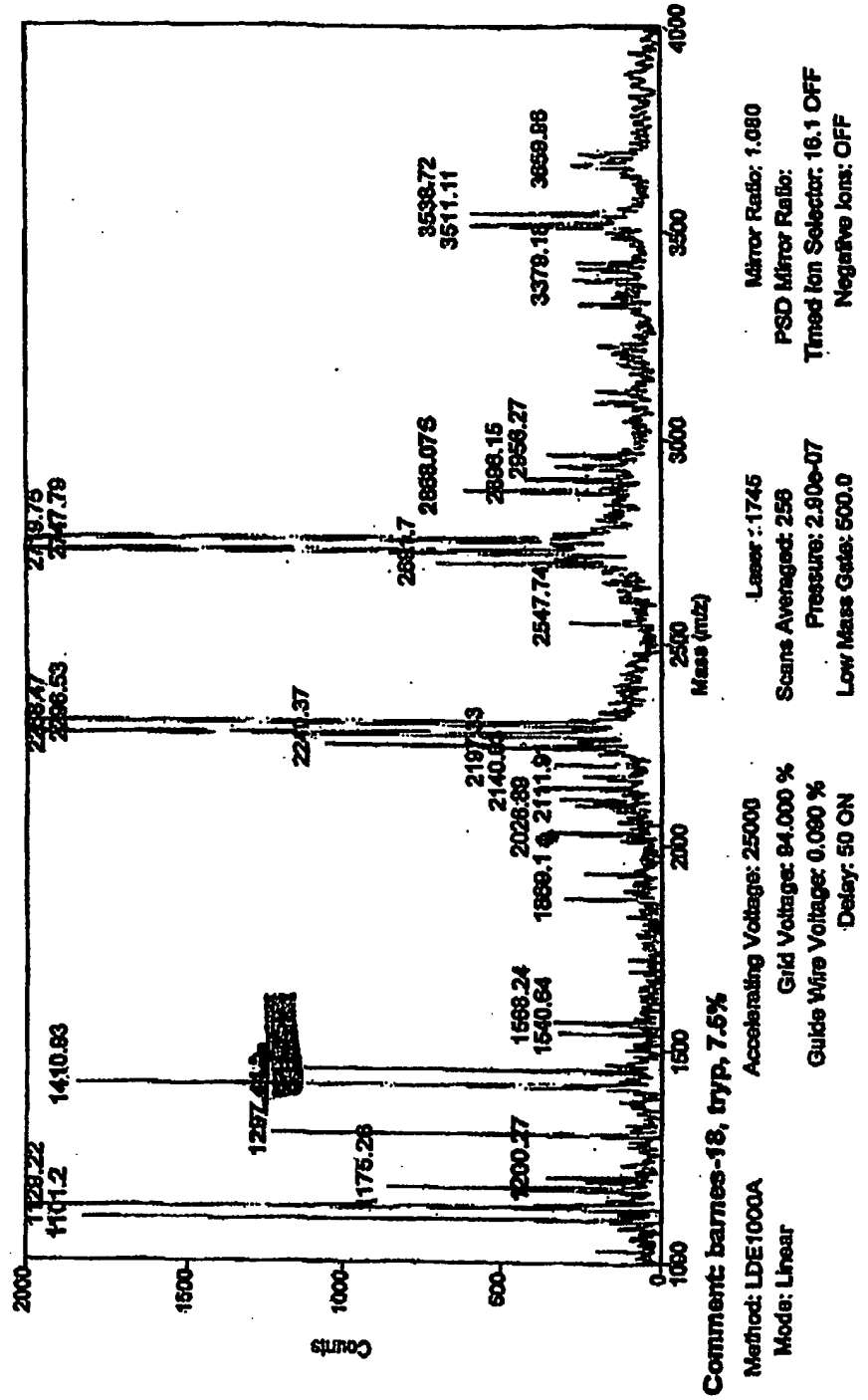


Figure 7L (Band 20)

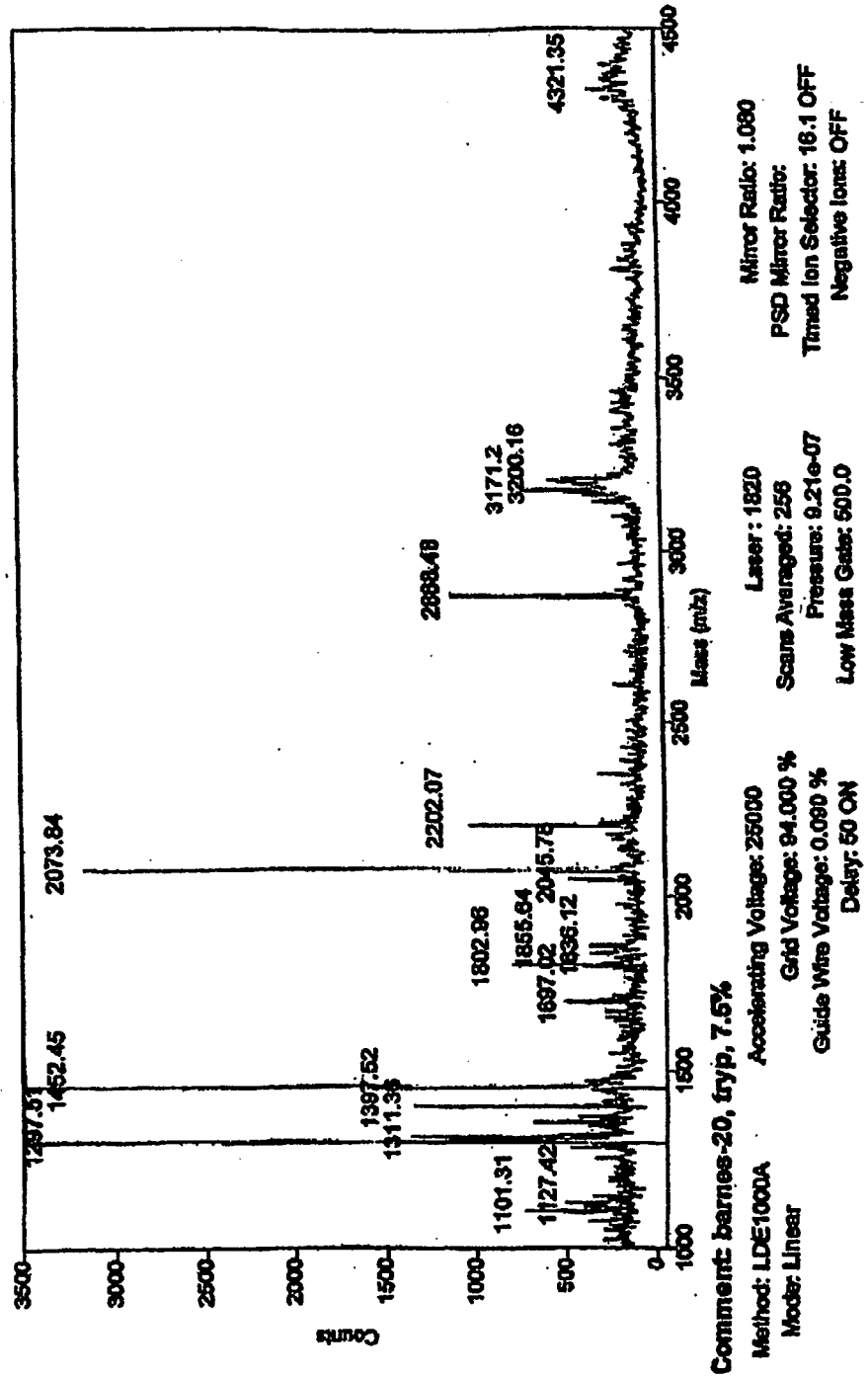


Figure 7M (Band 22)

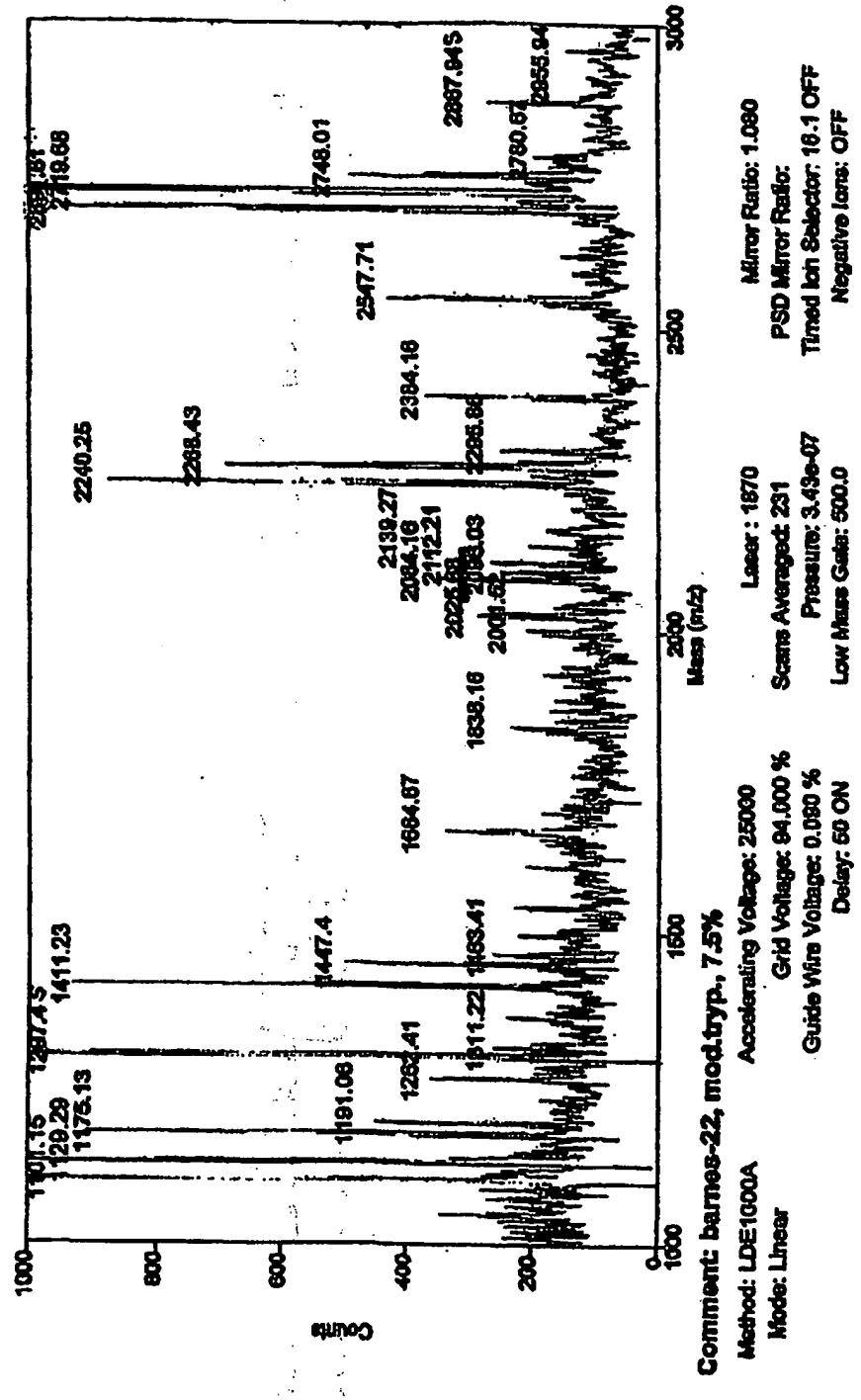


Figure 7N (Band 25)

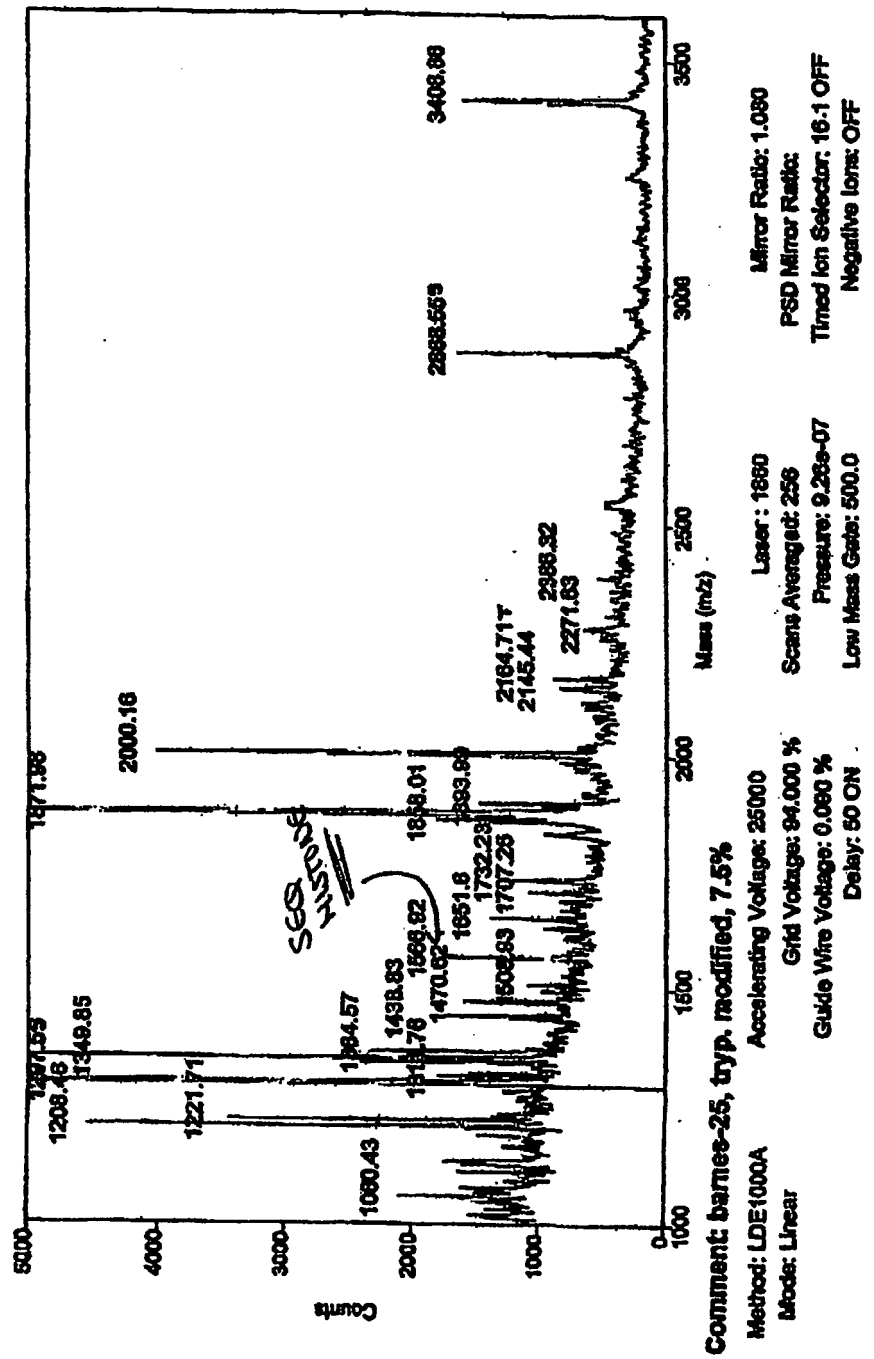
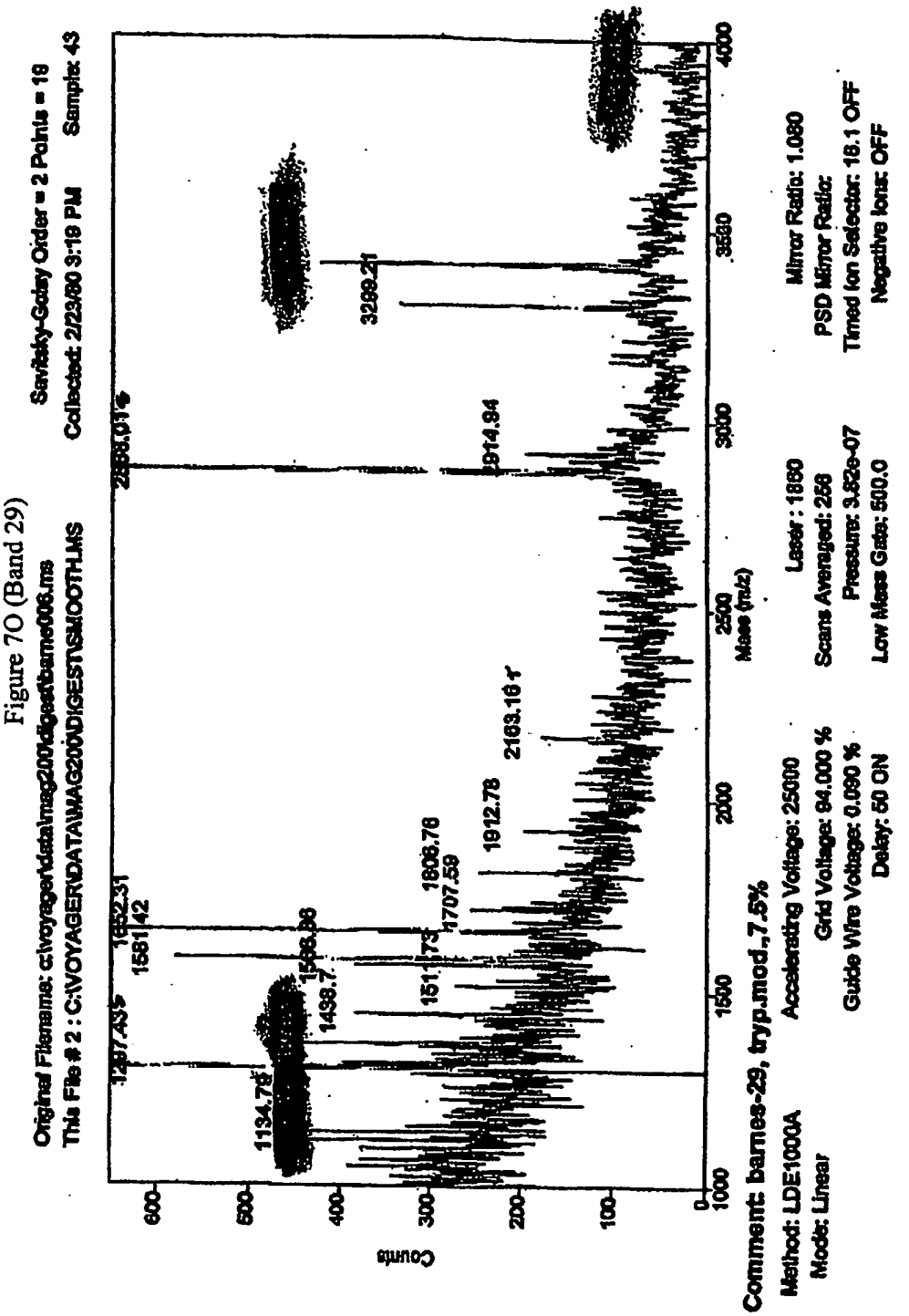


Figure 7O (Band 29)



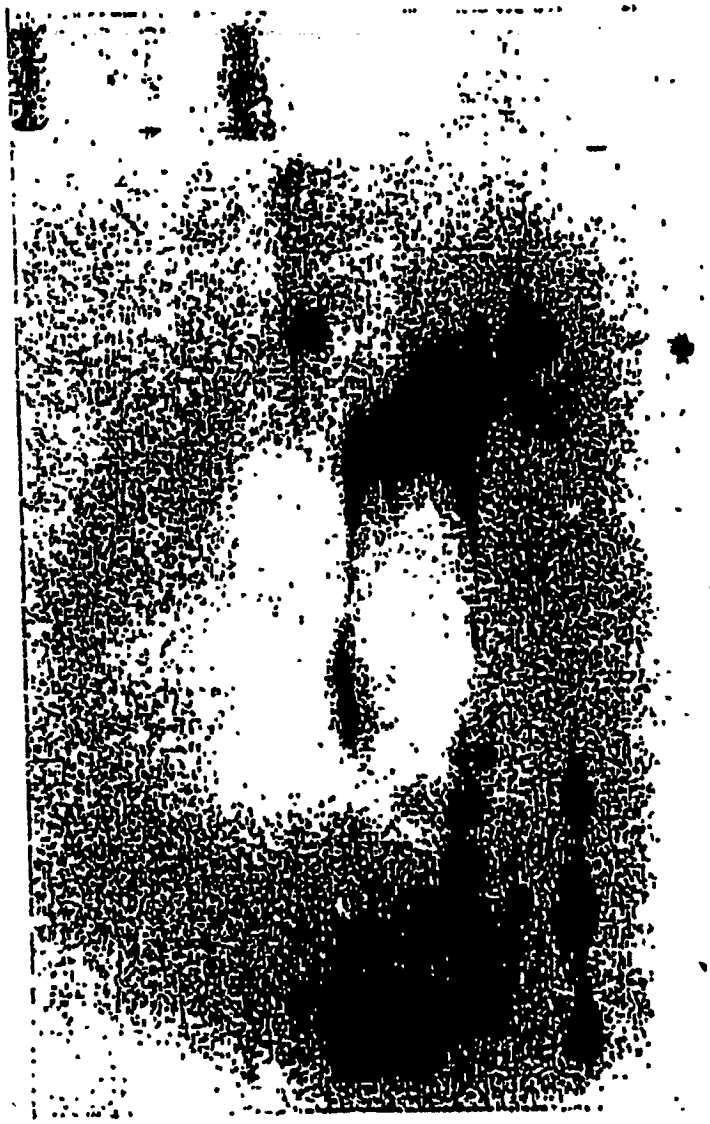


Figure 8

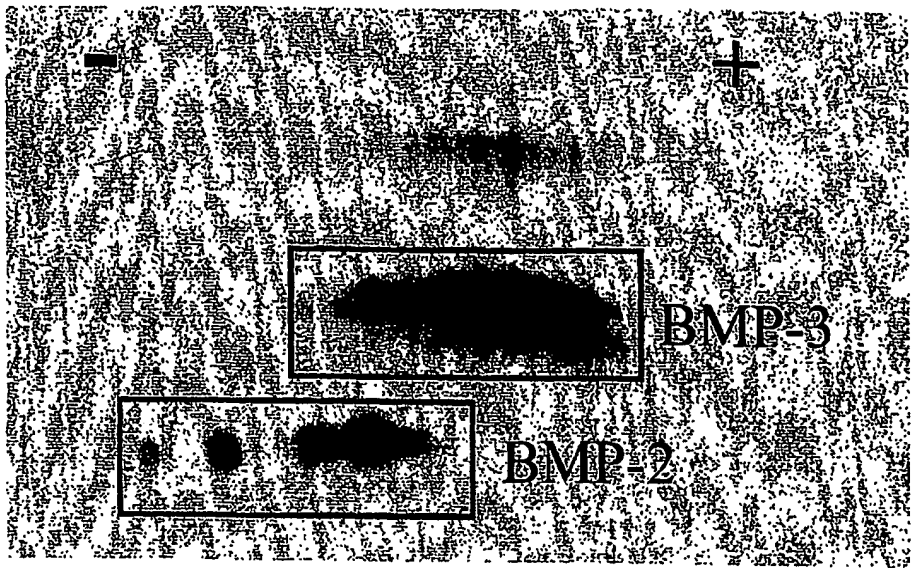


FIGURE 9A

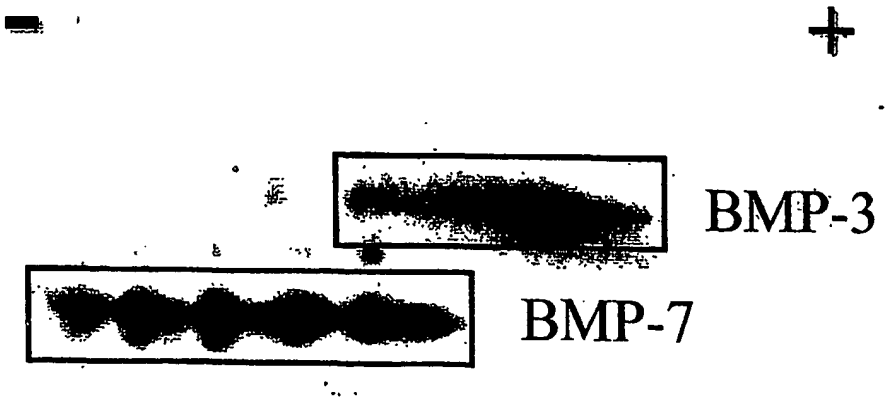


FIGURE 9B

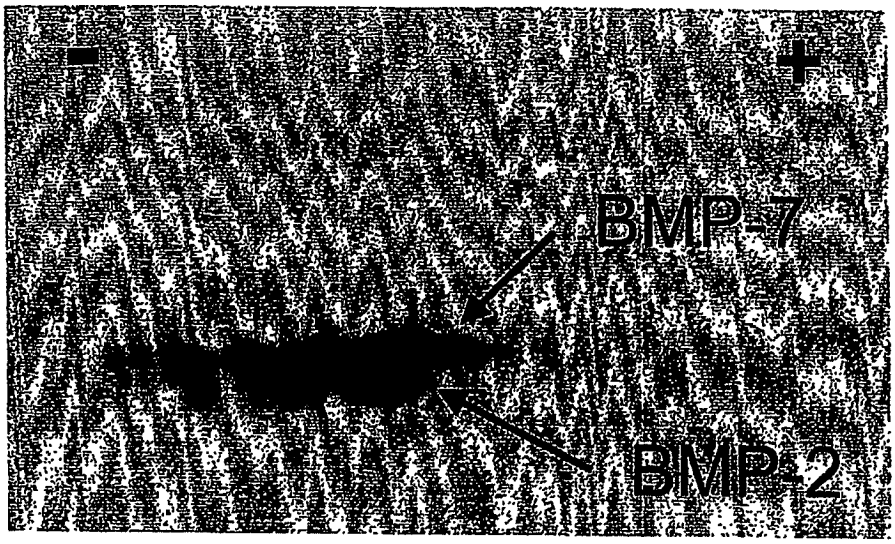


FIGURE 9C

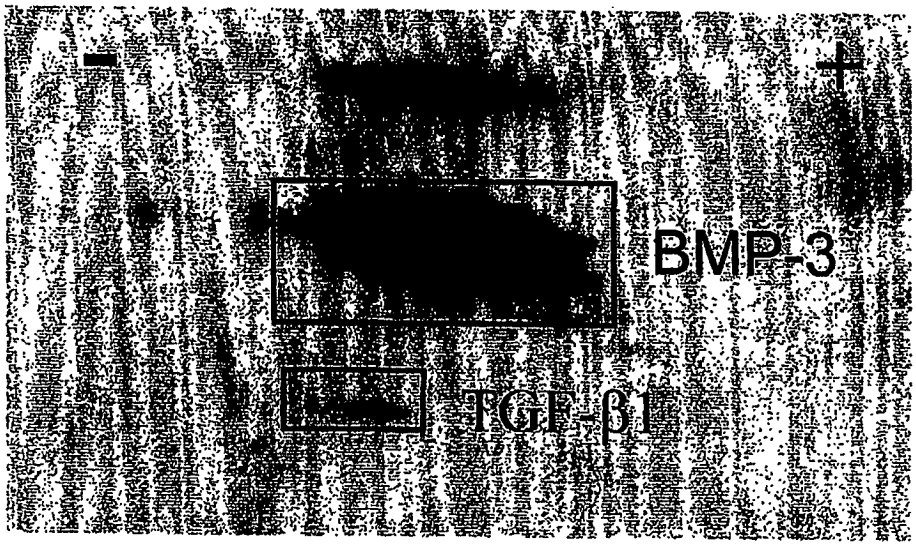


FIGURE 9D

FIGURE 10

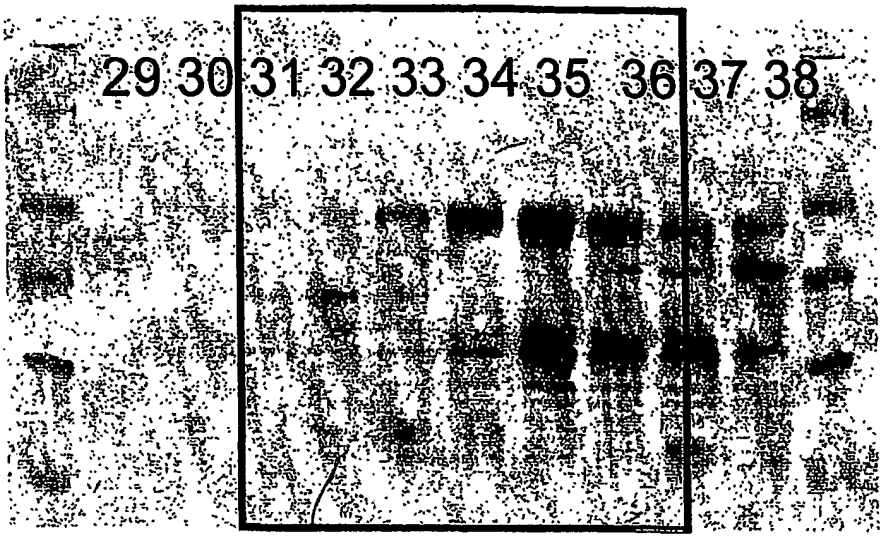


FIGURE 11

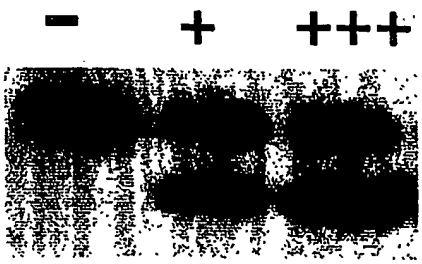
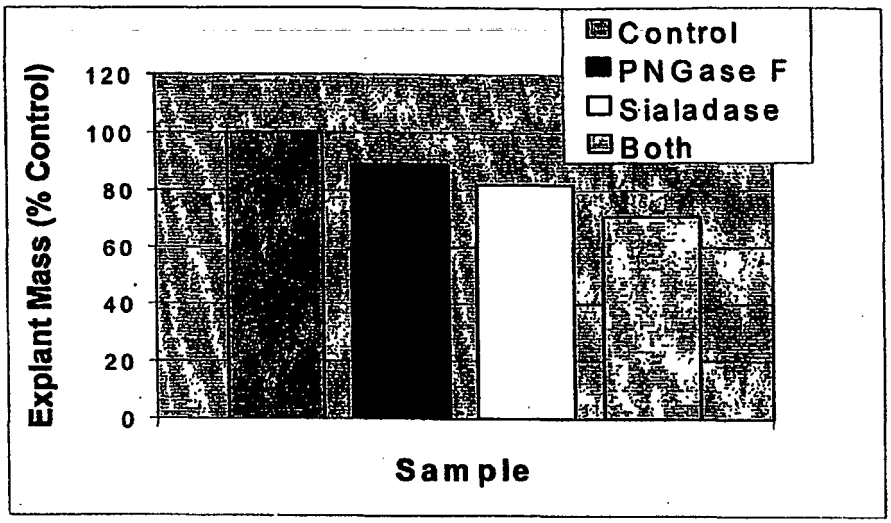


FIGURE 12

FIGURE 13A



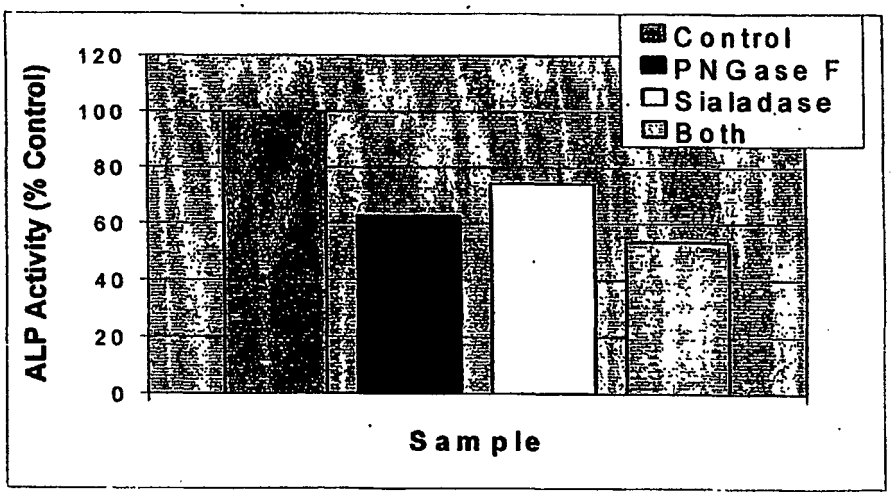


FIGURE 13B

FIGURE 14: Antibody Listing

Specificity	Antigen	Host Species	PC/MC	Source	Catalog No.
TGF-β1 (human)	Protein	Rabbit	Polyclonal	Promega	G1221
TGF-β2 (human)	Peptide	Rabbit	Polyclonal	Santa Cruz Biotechnology	sc-90
TGF-β3 (human)	Peptide	Rabbit	Polyclonal	Santa Cruz Biotechnology	sc-82
BMP-2 (human)	Protein	Rabbit	Polyclonal	Austral Biologics	PA-513-9
BMP-3 (human)	Peptide	Chicken	Polyclonal	Research Genetics	NA
BMP-4 (human)	Peptide	Goat	Polyclonal	Santa Cruz Biotechnology	so-6896
BMP-5 (human)	Peptide	Goat	Polyclonal	Santa Cruz Biotechnology	sc-7405
BMP-6 (human)	Peptide	Mouse	Monoclonal	Novocastra Laboratories	NCL-BMP6
BMP-7 (human)	Peptide	Rabbit	Polyclonal	Research Genetics	NA
FGF-1 (human)	Peptide	Goat	Polyclonal	Santa Cruz Biotechnology	sc-1884
osteonectin (bovine)	Protein	Mouse	Monoclonal	DSHB	AON-1
osteocalcin (bovine)	Protein	Rabbit	Polyclonal	Accurate Chemicals	A761/R1H
serum albumin (bovine)	Protein	Rabbit	Polyclonal	Chemicon International	AB870
transferrin (human)	Protein	Chicken	Polyclonal	Chemicon International	AB797
apo-A1 lipoprotein (human)	Protein	Goat	Polyclonal	Chemicon International	AB740

Figure15 A. Identification of Proteins by Amino Acid Sequencing of Tryptic Fragments

Band	Sample	Sequence Data	Best Database Match	Match	Identification	Species	Acc. No.	AAs
1								
2	fx 49 (1579)	XLAAAGYDVEK	ALAAAGYDVEK	11/11	histone H1.c	human	87668 (NCBI)	65-75
3	fx 67 (1346)	SLEKVCADLIR	SLEKVCADLIR	11/11	40s Ribosomal Protein S20	rat	R3RT20 (PIR)	31-41
4	fx 65 ()	(V)VCGMLGFPSEAPV	VVCGMLGFPGEKRV	11/14	LORP	mouse	AAC95338 (NCBI)	213-226
5	N terminal seq	STGVLLPLQNNELPG	STGVLLPLQNNELPG	15/15	BMP-3	human	4557371 (NCBI)	290-304
	fx 72 (3925)	STGVLLPLQNNELPGA EYQY	STGVLLPLQNNELPG AEYQY	20/20	BMP-3	human	4557371 (NCBI)	290-309
	fx 74 (3409)	STGVLLPLQ	STGVLLPLQ	9/9	BMP-3	human	4557371 (NCBI)	290-298
6	fx 55 (1566)	(S)QTLQFXE	SQTLQFDE	7/8	BMP-3	human	4557371 (NCBI)	346-353
	fx 47	VYAF	no match		???			
	N terminal seq	HAGKYSREKNT(P)A(P)	HGGKYSREKNQPKP	11/14	α2-Macroglobulin Receptor Assoc. Pro.	human	P30533 (Swiss-Prot)	31-46
	fx 57 (1438)	SQTLQFDEQ	SQTLQFDEQ	9/9	BMP-3	human	4557371 (NCBI)	346-354
	fx 57 (1652)	SLKPSNHA	SLKPSNHA	8/8	BMP-3	human	4557371 (NCBI)	410-417
7	fx 51 (1093)	AALRPLVKP	AALRPLVKP	9/9	60s Ribosomal Protein L32	mouse	P17932 (Swiss-Prot)	1-9
	fx 37 (no MS)	A(H)I(Q)VERYV	AIVER	5/5	60s Ribosomal Protein L32	mouse	P17932 (Swiss-Prot)	109-113
	fx 37 (no MS)	A(H)I(Q)VERYV	HQSDRYV	5/7	60s Ribosomal Protein L32	mouse	P17932 (Swiss-Prot)	22-28
8	fx 78 ()	XALF(G)AQLGXALGPI	no match		???			
9	fx 56 (1567)	SQTLQFDEQT	SQTLQFDEQT	10/10	BMP-3	human	P12645 (Swiss-Prot)	346-355

Figure 15 B. Identification of Proteins by Amino Acid Sequencing of Tryptic Fragments

Band	Sample	Sequence Data	Best Database Match	Match	Identification	Species	Acc. No.	AAs
11	fx 55 (1311)	SQTLXF	SQTLQF	5/6	BMP-3	human	4557371 (NCBI)	346-351
	fx 47 (1772)	VLATVTKPVGGDK	VLATVTKPVGGDK	13/13	60s Ribosomal Protein L6	human	Q02878 (Swiss-Prot)	87-99
	fx 76 (1795)	xVFAL	VFAL	4/4	60s Ribosomal Protein L6	human	Q02878 (Swiss-Prot)	273-276
	fx 61 (1145)	AVPQLQGYLR	AIPQLQGYLR	9/10	60s Ribosomal Protein L6	human	Q02878 (Swiss-Prot)	282-271
18								
22	fx 58 (1101)	ALDAAYCFR	ALDAAYCFR	9/9	TGF-β2	human	P08112 (Swiss-Prot)	303-311
	fx 69 (no match)	GYNANFCAGACPYL	GYNANFCAGACPYL	14/14	TGF-β2	human	P08112 (Swiss-Prot)	340-353
	fx 66 (1411.71)	VNSQSLSPY	VNSQSLSPY	9/9	SPP24	bovine	Q27987 (Swiss-Prot)	42-50
25	fx 39 (1470)	KAAKPSV(P)	KAAKPSVP	8/8	Histone H1.x	human	JC4928 (PIR)	199-206
29								

fx = fraction number (molecular weight of fragment, as measured by SDS-PAGE)

Figure16A. Identification of Proteins by Mass Spectrometry of Tryptic Fragments

Band	Mass Spec Profile	Species	Acc. No.	Mass Spec Data	Mass Spec Database	Mass Difference	AAs	% Coverage	Comments
1	4 peaks match with histone H1.c	human	87668 (NCBI)	1172.97	1172.37	0.60	110-121	22	15 MS peaks match with Band 2
				1579.87	1579.71	0.16	65-79		
				1708.47	1707.89	0.58	64-79		
				2011.58	2012.32	-0.74	35-54		
2	3 peaks match with histone H1.c	human	87668 (NCBI)	1579.76	1579.71	0.05	65-79[a]	16	identification of starred peptide confirmed by sequence analysis
				1708.02	1707.89	0.13	64-79		
				2012.12	2012.32	-0.20	35-54		15 MS peaks match with Band 1
3	7 peaks match with ribosome S20	rat	R3RT20 (PIR)	1129.76	1129.40	0.36	50-59	62	
				1156.21	1156.30	-0.09	76-83		
				1334.46	1334.62	-0.16	56-66		
				1352.13	1351.58	0.55	88-99		
				1518.04	1517.77	0.27	9-21		
				1919.02	1919.19	-0.17	5-21		
				3404.02	3404.87	-0.85	88-119		
4	3 peaks match with Lysyl Oxidase RP	human	NP002309 (Swiss-Prot)	1987.95	1988.27	-0.32	150-167	8	12 MS peaks match with Band 8
				2410.35	2410.63	-0.28	648-669		
				2610.57	2610.10	0.47	455-478		

Figure 16B. Identification of Proteins by Mass Spectrometry of Tryptic Fragments

Band	Mass Spec Profile	Species	Acc. No.	Mass Spec Data	Mass Spec Database	Mass Diff-erence	AAs	% Cover-age	Comments
5	9 peaks match with BMP-3	human	4557371 (NCBI)	1113.32	1113.31	0.01	361-368	48	% coverage calculation is relative to the mature BMP-3, 183 AAS (290-472)
				1438.53	1438.58	-0.05	346-357		
				1566.76	1566.76	0.00	345-357		
				1651.86	1651.91	-0.05	410-424		
				1794.09	1794.02	0.07	346-360		
				2268.46	2268.63	-0.17	374-392		
				2424.45	2424.81	-0.36	373-392		identification of starred peptide confirmed by sequence analysis
				3409.15	3407.77	1.38	290-318*		
6	3 peaks match with α2-Macroglobulin RAP	human	P30533 (Swiss-Prot)	1002.24	1002.15	0.09	283-290	17	
				2362.58	2362.43	0.15	129-150		
				3048.51	3048.52	-0.01	257-282		
	2 peaks match with BMP-3	human	4557371 (NCBI)	1566.93	1566.75	0.18	346-357	15	% coverage calculation is relative to the mature BMP-3, 183 AAS (290-472)
				1651.88	1651.91	-0.03	410-424		

Figure 16 C. Identification of Proteins by Mass Spectrometry of Tryptic Fragments

Band	Mass Spec Profile	Species	Acc. No.	Mass Spec Data	Mass Spec Database	Mass Difference	AAs	% Coverage	Comments
7	4 peaks match with ribosome L32	mouse	P17932 (Swiss-Prot)	1033.25	1033.17	0.08	67-75	33	
				1093.31	1093.40	-0.09	1-10*		
				1134.72	1134.28	0.44	65-74		
				1449.78	1449.66	0.12	19-29		
	5 peaks match with BMP-3	human	4557371 (NCBI)	1060.42	1060.20	0.22	102-111	21	% coverage calculation is relative to the mature BMP-3, 183 AAS (290-472)
				1113.39	1113.31	0.08	361-368		
				1360.26	1360.58	-0.32	190-200		
				1652.28	1651.91	0.37	410-424		
				1793.62	1794.02	-0.40	346-360		
8	1 peak matches with Lysyl Oxidase RP	human	NP002309 (Swiss-Prot)	2410.37	2410.63	-0.26	648-669	3	12 MS peaks match with Band 4
9	6 peaks match with BMP-3	human	4557371 (NCBI)	1113.14	1113.31	-0.17	361-368	36	% coverage calculation is relative to the mature BMP-3, 183 AAS (290-472)
				1438.60	1438.58	0.02	346-357		
				1566.77	1566.76	0.01	345-357		
				1651.91	1651.61	0.30	410-424		
				2901.67	2901.19	0.48	41-66		
				3408.94	3407.77	1.17	290-318		

Figure 18 D. Identification of Proteins by Mass Spectrometry of Tryptic Fragments

Band	Mass Spec Profile	Species	Acc. No.	Mass Spec Data	Mass Spec Database	Mass Difference	AAs	% Coverage	Comments
11	5 peaks match with BMP-3	human	4557371 (NCBI)	1113.23	1113.31	-0.08	361-368	48	% coverage calculation is relative to the mature BMP-3, 183 AAS (290-472)
				1651.73	1651.91	-0.18	410-424		
				1793.58	1794.02	-0.44	346-360		
				2424.24	2424.81	-0.57	373-392		
				3408.34	3407.77	0.57	290-318		
	5 peaks match with ribosome L6	human	Q02878 (Swiss-Prot)	1140.38	1140.23	0.15	114-122	16	
				1526.88	1526.86	0.02	141-155		
		mouse	P47911 (Swiss-Prot)	1059.15	1059.12	0.03	10-20		
				1145.36	1145.35	0.01	262-271		
				1386.74	1386.68	0.06	260-271		
18	4 peaks match with TGF-β2	human	P08112 (Swiss-Prot)	1101.20	1101.26	-0.06	303-311	52	
				1175.26	1175.42	-0.16	400-409		
				2240.37	2240.60	-0.23	312-328		
				2691.70	2691.91	-0.21	340-362		
	5 peaks match with SPP24	bovine	Q27987 (Swiss-Prot)	1410.93	1411.60	-0.67	42-53	30	
				1447.59	1447.65	-0.06	113-124		
				1540.64	1540.60	0.04	86-98		
				1869.10	1869.05	0.05	62-77		
				2268.47	2268.57	-0.10	33-53		

Figure 16 E. Identification of Proteins by Mass Spectrometry of Tryptic Fragments

Band	Mass Spec Profile	Species	Acc. No.	Mass Spec Data	Mass Spec Database	Mass Difference	AAs	% Coverage	Comments
22	5 peaks match with TGF-β2	human	P08112 (Swiss-Prot)	1101.15	1101.26	-0.11	303-311	63	
				1175.13	1175.42	-0.29	400-409		
				2084.16	2084.42	-0.26	312-347		
				2240.25	2240.60	-0.35	312-328		
				2691.61	2691.91	-0.30	340-362		
	2 peaks match with SPP24	bovine	Q27967 (Swiss-Prot)	1411.23	1411.60	-0.37	42-53	11	
				1447.40	1447.65	-0.25	113-124		
25	5 peaks match with histone H1.x	human	JC4928 (PIR)	1208.46	1208.40	0.06	48-57	14	
				1221.71	1222.35	-0.64	107-118		
				1348.85	1350.52	-0.67	107-119		
				1364.57	1364.59	-0.02	48-58		
				1732.23	1732.97	-0.74	43-57		
	5 peaks match with BMP-3	human	4557371 (NCBI)	1060.43	1060.20	0.23	102-111	31	% coverage calculation is relative to the mature BMP-3, 183 AAS (290-472)
				1438.83	1438.58	0.25	348-357		
				1566.92	1566.76	0.16	345-357		
				1651.80	1651.91	-0.11	410-424		
				3408.86	3407.77	1.09	290-318		

Figure 16 F. Identification of Proteins by Mass Spectrometry of Tryptic Fragments

Band	Mass Spec Profile	Species	Acc. No.	Mass Spec Data	Mass Spec Database	Mass Difference	AAs	% Coverage	Comments
28	4 peaks match with BMP-3	human	4557371 (NCBI)	1113.22	1113.31	-0.09	361-368	27	% coverage calculation is relative to the mature BMP-3, 183 AAS (290-472)
				1438.70	1438.68	0.12	346-357		
				1586.88	1586.75	0.11	345-357		
				3409.04	3407.77	1.27	290-318		

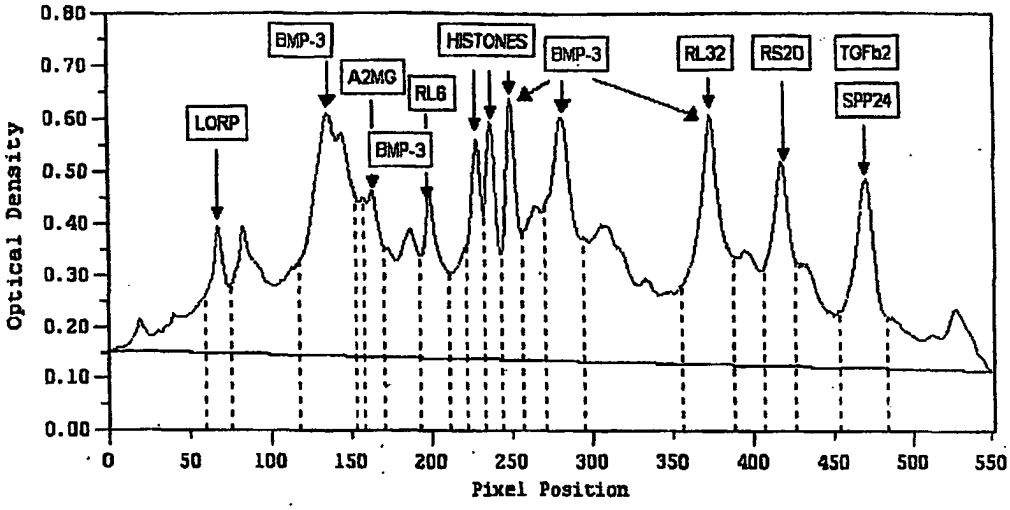


Figure 17A

Figure 17B

FIGURE 18: Quantitation of Identified BP proteins

Identified Protein	Percentage of Total Protein
LORP	2
BMP-3	11
BMP-3 and A2-MG	3
RL6 & BMP-3	4
Histone	3
Histone	3
Histone & BMP-3	4
BMP-3	8
RL32 & BMP-3	8
RS2D	5
SPP24 & TGF-β2	6
Total	58%

Figure 19A Identification of Proteinsby Mass Spectrometry of Fragments from 2D Gels

Spot	Digest	Mass Spec Profile	Species	Acc. No.	MS Peaks			AAs	% Coverage	Comments
					Data	Database	Diff			
1	Lys-C	2 peaks match with Coagulation Factor XIIIb	Human	P05160 (Swiss-Prot)	1837.01	1837.14	-0.13	472-487	8	
					1921.65	1921.14	0.51	366-382		
					2679.51	N/A	N/A	488-504		peptide match confirmed by sequence analysis
2	Trypsin	2 peaks match with LORP	Human	NP002309 (Swiss-Prot)	1609.57	1609.88	-0.31	241-253	5	
					2410.89	2410.63	0.26	648-669		
3	Lys-C	8 peaks match with Cathepsin L Precursor	Bovine	P25975 (Swiss-Prot)	1407.26	1406.80	0.46	105-116	41	
					1546.84	1546.70	0.14	58-70		
					1661.16	1660.80	0.36	21-33		
					1681.86	1680.80	1.06	301-314		
					1834.71	1834.00	0.71	318-334		
					2352.90	2351.50	1.40	274-295		
					2381.50	2380.70	0.80	239-261		
					2721.51	2721.10	0.41	131-154		

Figure 19B *Identification of Proteinsby Mass Spectrometry of Fragments from 2D Gels*

Spot	Digest	Mass Spec Profile	Species	Acc. No.	MS Peaks			AAs	% Coverage	Comments
					Data	Database	Diff			
4	Lys-C	2 peaks match with Lysyl Oxidase	Rat	P16836 (Swiss-Prot)	1461.58	N/A	N/A			peptide matches confirmed by sequence analysis
					4595.08	4593.06	2.02			
5	Lys-C	3 peaks match with TGF-b2	Bovine	P21214 (Swiss-Prot)	774.58	774.90	-0.34	26-31	20	
					809.67	809.94	-0.27	32-37		
					1175.26	1175.43	-0.17	98-107		
		2 peaks match with SPP24	Bovine	Q27987 (Swiss-Prot)	1415.56	1415.58	-0.02	42-60	16	
					2187.98	2187.51	0.47	21-32		
6	Trypsin	13 peaks match with SPP24	Bovine	Q27987 (Swiss-Prot)	1078.06	1078.15	-0.09	78-85	60	
					1101.07	1101.31	-0.24	99-108		
					1172.42	1172.31	0.11	99-108		
					1411.53	1411.60	-0.07	42-53		
					1447.63	1447.65	-0.02	113-124		
					1540.57	1540.52	0.05	86-98		
					1696.79	1696.71	0.08	85-98		
					1869.16	1869.05	0.11	62-77		
					2026.01	2025.24	0.77	61-77		
					2272.97	2272.56	0.41	21-41		
					2600.18	2599.65	0.53	78-98		
					2693.30	2693.81	-0.51	88-108		
					2928.80	2928.01	0.79	125-151		

Figure 19C Identification of Proteinsby Mass Spectrometry of Fragments from 2D Gels

Spot	Digest	Mass Spec Profile	Species	Acc. No.	MS Peaks			AAs	% Coverage	Comments
					Data	Database	Diff			
7	Lys-C	4 peaks match with TGF-b2	Bovine	P21214 (Swiss-Prot)	774.56	774.90	-0.34	26-31	42	
					809.69	809.94	-0.25	32-37		
					1175.12	1175.43	-0.31	98-107		
					3166.10	3166.66	1.44	1-25		
		1 peak matches with SPP24	Bovine	Q27967 (Swiss-Prot)	2167.77	2167.51	0.26	42-60	10	
8	Trypsin	12 peaks match with ribosome L3	Bovine	P39872 (Swiss-Prot)	917.38	917.14	0.25	348-355	37	
					984.23	984.15	0.08	10-18		
					1192.62	1192.40	0.22	286-296		
					1380.67	1380.65	0.02	249-260		
					1464.80	1464.63	0.17	103-114		
					1620.86	1620.82	0.04	103-115		
					1778.84	1779.00	-0.16	34-49		
					2238.43	2238.55	-0.12	30-49		
					2325.99	2325.65	0.34	177-197		
					2681.31	2681.04	0.27	200-223		
					2897.94	2898.43	-0.49	70-96		
					2946.10	2946.35	-0.25	198-223		

Figure 19D Identification of Proteinsby Mass Spectrometry of Fragments from 2D Gels

Spot	Digest	Mass Spec Profile	Species	Acc. No.	MS Peaks			AAs	% Coverage	Comments
					Data	Database	Diff			
9	Trypsin	7 peaks match with ribosome S3a	Mouse	P97351 (Swiss-Prot)	920.05	920.10	-0.05	19-26	29	
					1218.29	1218.31	-0.02	152-161		
					1346.62	1346.49	0.13	151-161		
					1516.69	1516.69	0.00	174-186		
					1593.72	1593.82	-0.10	94-108		
					1719.91	1720.00	-0.09	199-212		
					1953.12	1953.16	-0.04	65-81		
10	Trypsin	4 peaks match with histone H1.c	Human	87668 (NCBI)	1327.75	1327.56	0.19	34-46	23	
					1579.70	1579.71	-0.01	65-79		
					1707.65	1707.89	-0.24	64-79		
					2147.17	2147.53	-0.36	1-21		
11	Trypsin	6 peaks match with ribosome S4	Human	P12750 (Swiss-Prot)	1168.48	1168.38	0.10	230-239	23	
					1216.39	1216.39	0.00	134-144		
					1354.03	1353.61	0.42	230-241		
					1507.81	1507.69	0.12	198-210		
					1557.75	1557.98	-0.23	37-48		
					2140.34	2140.58	-0.24	221-239		
					2591.80	2591.90	-0.10	77-99		

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(54) Title: PROTEIN MIXTURES FOR WOUND HEALING

(57) Abstract: A protein mixture that is useful in the treatment of wounds, where the mixture is isolated from bone or is produced from recombinant proteins and may include two or more of BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, TGF-β1, TGF-β2, TGF-β3, and FGF-1.

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	WO 02 47713 A (SULZER BIOLOG INC) 20 June 2002 (2002-06-20) page 1, line 5 - line 6 page 11, line 1 - line 3 ---	1-17
X	US 5 290 763 A (POSER JAMES W ET AL) 1 March 1994 (1994-03-01) cited in the application the whole document ---	1-4,6,13
X	US 5 371 191 A (POSER JAMES W ET AL) 6 December 1994 (1994-12-06) the whole document ---	1-4,6,13
X	US 5 563 124 A (POSER JAMES W ET AL) 8 October 1996 (1996-10-08) cited in the application the whole document --- -/--	1-4,6,13

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Date of the actual completion of the international search	Date of mailing of the international search report
21 January 2003	29/01/2003

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int tional Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the retevant passages	Relevant to claim No.
X	US 6 054 122 A (DROHAN WILLIAM NASH ET AL) 25 April 2000 (2000-04-25) column 12, line 14 - line 53; example 10	1-4,6, 9-13
Y	US 5 116 738 A (ROSEN VICKI ET AL) 26 May 1992 (1992-05-26) column 1, line 27 - line 56	1-24
Y	US 5 141 905 A (ROSEN VICKI A ET AL) 25 August 1992 (1992-08-25) column 1 -column 3 column 6 -column 8 column 21, line 35 - line 37	1-24
Y	US 5 187 076 A (CELESTE ANTHONY J ET AL) 16 February 1993 (1993-02-16) column 7, line 35 -column 8, line 19 column 9, paragraph 2	1-24
Y	WO 96 41818 A (ELSON CLIVE ;TAYLOR JOHN (US); SINGH MANISH (US); DROHAN WILLIAM N) 27 December 1996 (1996-12-27) page 10, line 28 -page 11, line 2 page 23, line 16 - line 28 page 25, line 26 -page 27, line 29	1-24
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Y	US 5 356 630 A (GLOWACKI JULIANNE ET AL) 18 October 1994 (1994-10-18) claim 1	1-24

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 18-24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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